



1980

# The Short and Long Term Effects of Opiates on Myenteric Neurons

Peter J. Karras

Loyola University Chicago

## Recommended Citation

Karras, Peter J., "The Short and Long Term Effects of Opiates on Myenteric Neurons" (1980). *Dissertations*. Paper 1858.  
[http://ecommons.luc.edu/luc\\_diss/1858](http://ecommons.luc.edu/luc_diss/1858)

This Dissertation is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Dissertations by an authorized administrator of Loyola eCommons. For more information, please contact [ecommons@luc.edu](mailto:ecommons@luc.edu).



This work is licensed under a [Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License](https://creativecommons.org/licenses/by-nc-nd/3.0/).  
Copyright © 1980 Peter J. Karras

THE SHORT AND LONG TERM EFFECTS OF OPIATES  
ON MYENTERIC NEURONS

by

Peter J. Karras

A Dissertation Submitted to the Faculty of the Graduate School  
of Loyola University of Chicago in Partial Fulfillment  
of the Requirements for the Degree of  
Doctor of Philosophy

April 1980

## ACKNOWLEDGEMENTS

The author expresses appreciation and gratitude to Drs. R.A. North and G. Henderson for their guidance, patience and friendship. The author is also grateful to Drs. Alexander Karczmar, Herbert Proudfit, Bruce Wainer and Robert Wurster for their assistance in the preparation of this dissertation. Finally, the author would like to thank Mrs. Kayoko Kunitomo for her aid and kindness and Miss Gail Scheutzow for her excellent secretarial assistance.

## VITA

Peter J. Karras was born on October 8, 1953 in Chicago, Illinois.

He received his primary and secondary education in the Chicago public school system.

He was awarded the degree of Bachelor of Science in biology by Loyola University in June, 1975. In July, 1975 he was accepted by the Department of Pharmacology at Loyola University of Chicago, Stritch School of Medicine. The author was a Basic Science Fellow, NIH Pre-doctoral Trainee and an Arthur J. Schmitt fellow.

1. North, R.A. and Karras, P.J. (1978). Opiate tolerance and dependence induced in vitro in single myenteric neurons. *Nature* 272, 73-75.

2. Karras, P.J. and North, R.A. (1978). Opiate tolerance and dependence on single myenteric neurons. *Fedn. Proc.*, 37, 311.

3. North, R.A. and Karras, P.J. (1978). Tolerance and dependence in vitro. In Characteristics and Functions of Opioids. eds. van Ree, J.M. and Terenius, L. pp. 25-36. Amsterdam: Elsevier.

4. Karras, P.J. and North, R.A. (1979). Inhibition of neuronal firing by opiates: Evidence against the involvement of cyclic nucleotides. *Br. J. Pharmac.*, 65, 647-652.

## TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	ii
VITA	iii
LIST OF TABLES	viii
LIST OF FIGURES	ix
Chapter	
1. INTRODUCTION	1
1.1. OPIATE TOLERANCE AND DEPENDENCE	7
1.1.1. Definitions	8
1.1.2. Opiate Receptor Studies	10
1.1.3. Pharmacology of Opiates	14
1.1.4. Mechanisms and Theories of Opiate Tolerance and Dependence	21
1.2. SINGLE NEURON STUDIES	
1.2.1. The Acute Effects of Opiates	29
1.2.2. Chronic Effects of Opiates	34
1.3. INTERACTION OF CYCLIC-AMP AND RELATED COMPOUNDS WITH OPIATES	
1.3.1. Antinociception	38
1.3.2. Studies on Brain Homogenates and Neuro- blastoma-Glioma Hybrid Cells	39
1.3.3. Myenteric Plexus	41
1.4. THE MYENTERIC PLEXUS	
1.4.1. Anatomy	43
1.4.2. Electrophysiology	46
1.4.3. Actions of Opiates on the Guinea- Pig Myenteric Plexus	49
1.4.4. Opiate Receptor Studies	51
1.4.5. Opiate Tolerance and Dependence	53
1.5. SPECIFIC AIMS	60

## 2. METHODS

2.1.	THE TISSUE	61
2.2.	IMMOBILIZATION	62
2.3.	PERFUSION AND DRUG APPLICATION. DRUGS USED	63
2.4.	THE TISSUE BATH	65
2.5.	ELECTROPHYSIOLOGICAL RECORDINGS	66
2.6.	TWENTY-FOUR HOUR INCUBATIONS ( <u>IN VITRO</u> )	68
2.7.	MORPHINE PELLET IMPLANTED OR INJECTED ANIMALS ( <u>IN VIVO</u> )	70
2.8.	PLASMA LEVELS OF MORPHINE	71
2.9.	DEFINITIONS	72
2.1.0.	ANALYSIS OF DATA	73

## 3. RESULTS

3.1.	EXTRACELLULAR RECORDING	74
3.2.	THE ACTION OF OPIATES ON SINGLE UNIT ACTIVITY	
3.2.1.	Morphine	80
3.2.2.	Normorphine	80
3.2.3.	Levorphanol	83
3.2.4.	Dextrorphan	88
3.2.5.	Cyclazocine	88
3.2.6.	Ketocyclazocine	91
3.2.7.	Buprenorphine	91
3.2.8.	Narcotic Antagonists	92
3.3.	THE ACTION OF CYCLIC NUCLEOTIDES AND RELATED COMPOUNDS ON SINGLE UNIT ACTIVITY	
3.3.1.	Cyclic-AMP	102
3.3.2.	Dbcyclic-AMP	102
3.3.3.	PGE <sub>2</sub>	103
3.3.4.	IBMX	108
3.4.	THE LACK OF INVOLVEMENT OF CYCLIC NUCLEOTIDES IN THE INHIBITION OF NEURONAL FIRING BY OPIATES	
3.4.1.	Cyclic-AMP	109
3.4.2.	Dbcyclic-AMP	109
3.4.3.	PGE <sub>2</sub>	112
3.4.4.	IBMX	112

3.5.	EFFECTS OF PROLONGED EXPOSURE TO OPIATES	
3.5.1.	Prolonged Exposures (1 h) Whilst Recording from a Single Neuron	121
3.5.2.	Prolonged Exposures (24 h) Prior to and During the Recording from a Single Neuron	
3.5.2.1.	Normal Krebs Solution	121
3.5.2.2.	Morphine	127
3.5.2.3.	Levorphanol	128
3.5.2.4.	Dextrorphan	133
3.5.2.5.	Buprenorphine	136
3.5.2.6.	Ketocyclazocine	137
3.5.3.	Prolonged Exposure (3 days) <u>in Vivo</u>	145
3.6.	TIME COURSE IN THE RECOVERY FROM OPIATE TOLERANCE AND 'DEPENDENCE'	155
3.7.	THE EFFECT OF SEVERAL AGENTS ON THE DEVELOPMENT OF OPIATE TOLERANCE AND 'DEPENDENCE' AT THE SINGLE NEURON LEVEL	
3.7.1.	Synaptic Transmission Blockade	171
3.7.1.1.	Hexamethonium and Hyoscine	171
3.7.1.2.	Lidocaine	172
3.7.2.	Protein Synthesis Inhibitors	180
3.7.3.	Cyclic Nucleotides	181
3.7.3.1.	IBMX and Dbcyclic-AMP	181
4.	DISCUSSION	
4.1.	ACUTE ACTIONS OF OPIATES ON THE SPONTANEOUS ACTIVITY OF SINGLE MYENTERIC NEURONS	183
4.2.	THE INVOLVEMENT OF CYCLIC-AMP IN THE ACUTE INHIBITORY ACTION OF OPIATES ON NEURONAL FIRING	
4.2.1.	Inhibition of Neuronal Firing	187
4.2.2.	Inhibition of Acetylcholine Release	189
4.2.3.	Conclusion	190
4.3.	PROLONGED EXPOSURE TO OPIATES <u>IN VIVO</u> OR <u>IN VITRO</u>	192
4.3.1.	Tolerance to the Effect of Morphine	193
4.3.2.	Manifestation of 'Dependence' -- Effect of Naloxone	194
4.3.3.	Time Course of the Recovery from Opiate Tolerance and 'Dependence'	198

4.3.4.	<u>In Vitro</u> and <u>In Vivo</u> Exposure to Morphine -- A Comparison of Effects	200
4.3.5.	Opiate Tolerance and Dependence in other <u>In Vitro</u> Systems	202
4.3.6.	Alteration of the Development of Opiate Tolerance and 'Dependence' -- Concomitant Incubations with other Drugs	205
4.3.7.	Cellular Mechanism of Opiate 'Dependence' Speculation -- Enhanced Neuronal Excitability	208
4.3.8.	General Conclusions	210
4.4.	EXTRACELLULAR RECORDINGS OF MYENTERIC NEURONS -- LIMITATIONS	212
4.5.	PROPOSALS FOR FUTURE RESEARCH	213
	REFERENCES	214



# LIST OF TABLES

Table	Page
1. The Action of Morphine and Naloxone Studied in Neurons of Drug Naive and Opiate Pretreated Animals	36
2. The Number of Neurons Tested and Action of Several Narcotic Analgesics	90
3. The Action of Narcotic Agonists and Antagonists on Myenteric Neurons which have been Incubated 24 hours	126
4. A Comparison of the Effects of Narcotic Agonists and Antagonists on Myenteric Neurons Incubated 24 hours in a Morphine Solution and Those Removed from Morphine Pretreated Guinea-Pigs	148
5. Recovery from Opiate Tolerance in Single Myenteric Neurons	157
6. The Action of Naloxone on Myenteric Neurons Undergoing Withdrawal from Morphine	164

## LIST OF FIGURES

FIGURE	PAGE
1. Comparison of Neuronal and Smooth Muscle Action Potentials	77
2. Neuronal Spike Waveform Recorded Extracellularly	79
3. Inhibition of Neuronal Firing by Morphine and the Reversal by a Morphine Antibody	82
4. Concentration Dependent Inhibition in Firing of a Myenteric Neuron Produced by Normorphine	85
5. Effect of Morphine, Levorphanol, Dextrorphan and Naloxone on the Firing Rate of a Myenteric Neuron	87
6. Comparison of the Inhibitions in Firing Produced by Normorphine and Ketocyclazocine on a Myenteric Neuron	94
7. Comparison of the Effects Produced by Normorphine and Buprenorphine on the Firing Rate of a Myenteric Neuron	96
8. Inhibitions in Firing of a Myenteric Neuron by Morphine and Levorphanol and the Reversal of This Inhibition by Naloxone	99
9. Inhibition in Firing Rate Produced by Cyclic Adenosine-3',5'-Monophosphate and the Reversal by Theophylline	105
10. Concentration Dependent Inhibition in Neuronal Firing Produced by Dibutryl Cyclic Adenosine-3',5'-Monophosphate	107
11. The Lack of Effect of Dibutryl Cyclic Adenosine 3', 5'-Monophosphate on the Inhibition of Firing of a Myenteric Neuron Produced by Normorphine	111
12. The Interaction of Prostaglandin E <sub>2</sub> and Normorphine on the Firing Rate of a Myenteric Neuron	114
13. The Interaction of Prostaglandin E <sub>2</sub> and Dibutryl Cyclic Adenosine-3',5'-Monophosphate with the Inhibition of Neuronal Firing Produced by Normorphine	116

14.	The Lack of Effect of Isobutylmethylxanthine on the Inhibition of Firing of a Myenteric Neuron Produced by Morphine	118
15.	Inhibition in Firing Rate of a Myenteric Neuron by Normorphine	123
16.	The Effect of Prolonged Exposure to Morphine on the Firing Rate of a Myenteric Neuron	130
17.	The Effect of the Narcotic Antagonist Mr 2266 on the Firing Rate of a Myenteric Neuron Exposed to Levorphanol or Dextrorphan for 24 hours	135
18.	The Effect on a Myenteric Neuron of Prolonged Exposure to Buprenorphine	140
19.	The Effect on a Myenteric Neuron of Prolonged Exposure to Ketocyclazocine	142
20.	The Effect of Normorphine and Naloxone on the Firing Rate of Myenteric Neurons Removed from Morphine Pretreated Guinea-Pigs	150
21.	The Effect of Repeated Applications of Naloxone on the Firing Rate of Myenteric Neurons Removed from Morphine Pretreated Guinea-Pigs	154
22.	The Effect of Morphine on the Firing Rate of Myenteric Neurons Undergoing Opiate Withdrawal	159
23.	The Effect of Prolonged Exposures to Normorphine on the Firing Rate of Myenteric Neurons Undergoing Opiate Withdrawal	166
24.	The Effect of Repeated Applications of Naloxone on the Firing Rate of Myenteric Neurons Undergoing Opiate Withdrawal	170
25.	The Effect on a Myenteric Neuron of Prolonged Exposure to Morphine in Conditions of Synaptic Transmission Blockade	175
26.	The Effect on a Myenteric Neuron of Prolonged Exposure to Lidocaine	179

## 1. INTRODUCTION

The primary therapeutic use of morphine is to alleviate pain. Recent evidence suggests that opiates can exert their analgesic effect in three ways. First, opiates can inhibit primary afferent transmission in the dorsal horn of the spinal cord. Cells in lamina I and V of the spinal cord which receive noxious input are inhibited by morphine: whether they are firing spontaneously, or induced to fire by noxious stimuli applied to their receptive field (Kitahata, Kosaka, Taub, Bonikos and Hoffert, 1974; Takagi, Doi and Akaike, 1976; Duggan, Hall and Headley, 1977b). The response of cells in the spinal cord to non-noxious stimuli (jet of air, light touch) are not inhibited by morphine (Calvillo, Henry and Neuman, 1974). Further support for a direct spinal action of opiates includes the finding by Yaksh and Rudy (1976) that morphine injected into the spinal subarachoid space of a rat (with its spinal cord transected) produces antinociception. The response was rapid, making unlikely the possibility that diffusion of the opiate to supraspinal structures was involved. Second, opiates can inhibit somatosensory afferent transmission at the supraspinal level. Morphine applied directly to the periaqueductal grey (PAG) hypothalamus, nucleus reticularis gigantocellularis and medial thalamus results in analgesia (Herz, Albus, Metys, Schubert and Teschemacher, 1970; Pert and Yaksh, 1974; Takagi et al., 1976; Pert, 1978). One important observation concerning brain regions that appear to mediate opiate analgesia is that they receive input from the anterolateral pathway of the spinal cord:

a pathway which presumably carries pain information. Injection of morphine into certain other areas of the brain such as the cerebral cortex, tectum and midbrain reticular formation was ineffective in producing analgesia (Pert, 1978). However, interpretations of these findings are difficult in view of the possibility that morphine could have diffused into the ventricular system. Third, opiates can activate the descending inhibitory pathways. Descending pathways from the raphe magnus, periaqueductal grey and possibly the locus coeruleus inhibit the firing of dorsal horn cells and their responses to noxious input (Oliveras, Besson, Guilbaud and Liebeskind, 1974; Fields, Basbaum, Clanton and Anderson, 1977; Basbaum and Fields, 1978). Subsequent studies have shown that systemically administered morphine increases the spontaneous activity of cells in the raphe magnus and PAG (Anderson, Basbaum and Fields, 1977; Urca and Liebeskind, 1979). Satoh and coworkers (1971) presented additional support for the importance of descending supraspinal control by demonstrating that bradykinin induced increases in activity of dorsal horn cells were inhibited by morphine (2 mg/kg) in intact rabbits, but not in spinal rabbits.

With chronic exposure to morphine, tolerance to many of the acute effects develop and the sudden discontinuation of the drug leads to profound physiological and behavioral changes. Two methods have been used to establish the brain regions important in the development of opiate dependence. One method involves the lesioning of discrete brain regions. Chemical or electrolytic lesions attenuate or abolish certain signs of opiate withdrawal. The following areas have been lesioned in the rat and shown to be involved: ventromedial nucleus of

the hypothalamus (Kerr and Pozuelo, 1971), medial forebrain bundle (Glick and Charap, 1973), noradrenergic bundles which innervate the thalamus or hypothalamus (Lewis, Costa and Jacobowitz, 1976), medial thalamic nuclei (Teitelbaum, Catravas and McFarland, 1974) and caudate nucleus (Borison, 1971). In addition, it has been demonstrated in human addicts that prefrontal lobotomy reduces the craving for opiates (Mason and Hamby, 1948). It is difficult to assess any causal relationship from such studies: the lesions may owe their effects to nonspecific sequelae of the operation or to loss of integrated cerebral activity and circuitry which are essential to the expression of the opiate withdrawal syndrome. The second method involves the microinjection of narcotic antagonists into discrete brain sites. The injection of naloxone into a number of brain regions precipitates abstinence in addicted rats. The brain sites in which these withdrawal signs could be elicited appeared to be localized in the nucleus anterior ventralis, anterior medialis and parafascicularis of the thalamus (Wei, Loh and Way, 1973). More recently, Wei and coworkers (1975) reported that injection of naloxone into the medial hypothalamus and periaqueductal grey matter elicited an intense withdrawal reaction in morphine dependent rabbits, and concluded that the medullary and pontine regions of the brainstem were important areas for the development of opiate dependence. Although there appeared to be site specificity with the microinjection studies, it is difficult to reach definite conclusions due to incomplete information about the spread of the injected antagonist. Both the lesion and microinjection studies suggest a number of different brain sites as foci for addictive mechanisms; however, both studies suffer

from the inherent assumption (which need not necessarily be correct) that those areas which can elicit or suppress signs of withdrawal are those areas intimately involved in the development of dependence. In other words, it is not possible to decide whether the site of action was made at the primary site of withdrawal or along a complex neuronal circuit.

The basic mechanisms underlying the acute analgesic effect of opiates and opiate tolerance and addiction remain obscure in spite of numerous efforts by investigators using a variety of biochemical, behavioral and pharmacological techniques. It has been established that opiates act directly on neurons and the result of such an interaction are changes in the membrane properties of the neuron. It was my intention to elucidate the underlying mechanism of acute and chronic opiate action in single myenteric neurons using an electrophysiological approach.

Electrophysiological studies of neural tissue may be carried out in vivo or in vitro. There are advantages and disadvantages to each approach. Studies in vivo are performed under physiological conditions in which the anatomical and functional connections are left intact. Further advantage includes the ability to record while the animal is moving and behaving normally. Yet, there are limitations to the in vivo approach. The method of drug administration is usually by local application onto the tissue under investigation (iontophoresis, microinjection) or by systemic injections into blood vessels. In either case the concentration of the drug and the exact site of drug action is unknown. Advantages of the in vitro technique include the following.

One, the concentration of the drug at the site of its action is known. Two, the ionic composition of the perfusate can be altered when the ionic mechanism of action is to be studied. Three, it can generally be distinguished whether the observed effects are taking place directly on the neuron under study. Limitations of the technique include maintaining viability and stability for long periods of time and extrapolation of the data to the whole animal is difficult because anatomical connections have been severed and the physiological milieu altered.

The isolated myenteric plexus-longitudinal muscle of the guinea-pig ileum is a site in the peripheral nervous system which is sensitive to the opiates. It is a network of neurons adherent to the longitudinal muscle layer of the gut wall. The myenteric plexus affords one the opportunity to measure electrophysiologically the activity of single mammalian neurons which are morphine sensitive. The tissue provides a convenient model to study the responses which directly follow the interaction of a narcotic agonist with the opiate receptor. The acute actions of opiates in this preparation are to inhibit the nerve mediated contraction of the longitudinal muscle by reducing the output of acetylcholine from cholinergic nerves and to inhibit the spontaneous firing of neurons which can be recorded with extracellular electrodes (Paton, 1957; Schaumann, 1957; Sato, Takayanagi and Takagi, 1973; 1974; Dingledine, Goldstein and Kendig, 1974; Dingledine and Goldstein, 1975; 1976; North and Williams, 1977). That the action of opiates on the myenteric plexus is mediated by opiate receptors identical to those subserving analgesia in the central nervous system (CNS) is substantiated by the following evidence. First, the myenteric plexus possesses



opiate binding sites (Pert and Snyder, 1973). Second, the potency of an antagonist to inhibit naloxone binding in brain homogenates correlates well with its dissociation constant for binding in the myenteric plexus (Creese and Snyder, 1975). Third, the opiate action is stereospecific and occurs at low concentrations. Fourth, the action of opiates is reversed with a concentration of naloxone several fold lower than that of the agonist (Kosterlitz et al., 1968; North and Williams, 1977). Fifth, the relative potencies of a series of narcotic agonists in depressing the electrically induced contractions of the myenteric plexus-longitudinal muscle preparation are comparable to their analgesic potency in man (Kosterlitz and Waterfield, 1975; Creese and Snyder, 1975). Sixth, narcotics inhibit the spontaneous firing of myenteric neurons (Sato et al., 1973; Dingledine and Goldstein, 1975) which is similar to the action of opiates on central neurons (see review by North, 1979a). Seventh, the antagonist potency of narcotics determined in morphine-dependent monkeys correlates well with their antagonist potency determined on the guinea-pig ileum (Kosterlitz, Waterfield and Berthoud, 1974).

### 1.1. OPIATE TOLERANCE AND DEPENDENCE

When morphine is acutely administered to man a number of pharmacological effects are seen. Among the most salient acute effects are sedation, analgesia, euphoria, constipation, respiratory depression and cardiovascular depression. With repeated (chronic) exposure to morphine, tolerance to most of the acute effects develop. That is, to attain a desired effect the dose of the narcotic must be progressively increased. Chronic administration of morphine leads to profound physiological and behavioral changes which readily become evident upon the sudden discontinuation of the drug. The characteristic withdrawal syndrome which ensues has many of its clinical manifestations in directions opposite to those seen when opiates are given acutely (Jaffe, 1968). A more violent yet shorter lasting withdrawal syndrome is induced when naloxone or nalorphine is administered to chronically opiate treated individuals (Jaffe, 1968).

### 1.1.1. Definitions

Legal involvement in dealing with narcotic addiction along with common street terminology has led to much confusion over the exact definition of the narcotic related terms. Efforts have been made by the World Health Organization (WHO) to develop a consistent terminology with an exact definition. The following terms are commonly referred to in this manuscript and their WHO definition is presented (Isbell and Chrusciel, 1970).

**Drug Dependence:** A state, psychic and sometimes also physical, resulting from the interaction between a living organism and a drug, characterized by behavioral and other responses that always include a compulsion to take the drug on a continuous or periodic basis in order to experience its psychic effects, and sometimes to avoid the discomfort of its absence. Tolerance may or may not be present. A person may be dependent on more than one drug.

**Psychic Dependence:** A compulsion that requires periodic or continuous administration of a drug to produce pleasure or avoid discomfort. This compulsion is the most powerful factor in chronic intoxicification with psychotropic drugs, and with certain types of drugs may be the only factor involved in the perpetuation of abuse even in the case of most intensive craving. Psychic dependence, therefore, is the universal characteristic of drug dependence. Operationally, it is recognized by the fact that the dependent continues to take the drug in spite of conscious admission that it is causing harm to his health and to his social and familial adjustment, and that he takes great risks to obtain and maintain his supply of the drug.

**Physical dependence:** A pathological state brought about by repeated administration of a drug and that leads to the appearance of a characteristic and specific group of symptoms, termed an abstinence syndrome, when the administration of the drug is discontinued or -- in the case of certain drugs -- significantly reduced. In order to prevent the appearance of an abstinence syndrome the continuous taking of the drug is required. Physical dependence is a powerful factor in reinforcing psychic dependence upon continuing drug use or in relapse to drug use after withdrawal.

Tolerance: The state in which repetition of the same dose of a drug has progressively less effect, or in which the dose needs to be increased to obtain the same degree of pharmacological effect as was caused by the original dose.

Cross-tolerance: The state in which tolerance to one drug has the effect of causing tolerance to another drug of the same or a different chemical type.

### 1.1.2. Opiate Receptor Studies

The concept of receptors was originally introduced by Paul Ehrlich who stated that "substances can only be anchored at any particular part of the organism if they fit into the molecules of the recipient complex like a piece of mosaic finds its place in a pattern" (Ehrlich, 1960). However, several criteria must be fulfilled before a binding site can be classified as an opiate receptor. The drug must have a high affinity for the binding site, there must be a difference in the affinity with which stereoisomers bind, the number of binding sites must be limited, and interaction of an agonist with the binding site must elicit a biological response.

The interaction of opiates with high affinity membrane binding sites (receptors) results in a series of neuronal events leading to changes in pain perception, motor behavior, mood and autonomic responses. Opiate receptors have been localized to discrete brain areas; some probably subserve pain perception but others are not associated with pain.

Two techniques have been used to study opiate specific binding sites (receptors) in nervous tissue. The radioreceptor assay is an in vitro technique which discriminates between opiate enantiomers (Pert and Synder, 1973). Radioreceptor studies have revealed that the following brain regions are rich in opiate stereospecific binding sites: caudate, putamen, amygdala, periaqueductal grey, thalamus, hypothalamus and substantia gelatinosa (Kuhar, Pert and Synder, 1973; Lamotte, Pert

and Snyder, 1976). Autoradiography is the second technique available with which to study opiate specific binding sites. After parenteral injection,  $^3\text{H}$ -diprenorphine is localized primarily in the caudate, putamen, locus coeruleus, amygdala, zona compacta of the substantia nigra, nucleus tractus solitarius, nucleus ambiguus and the substantia gelatinosa (Pert, Kuhar and Snyder, 1975; Atweh, Murrin and Kuhar, 1978). There is a good correlation between the regional distribution of the specific binding sites indicated by the two techniques. Both techniques reveal negligible density of specific binding sites in the cerebellum and white matter. There are disadvantages and difficulties with each technique. The radioreceptor technique does not localize receptors in their physiological or functional setting and stereospecific binding has been shown to occur at clearly non-functional sites, such as glass filters (Snyder, Pasternak and Pert, 1975) and cerebroside sulfate (in vitro) (Loh, Cho, Wu and Way, 1974). There are also limitations with the autoradiographic technique. These include diffusion of the high affinity ligand away from the specific binding site to other areas which therefore sets limits to the resolution of the technique. Furthermore, the technique fails to differentiate between multiple high affinity binding sites. Also one is restricted to using high affinity compounds which are not readily metabolized by the body.

Subcellular distribution studies reveal high densities of stereospecific binding sites in the mitochondrial and microsomal brain fractions. These fractions are rich in synaptosomes and membrane fragments, respectively and the inference is that opiate receptors may be located on nerve

terminals (Pert, Snowman and Snyder, 1974). Evidence that opiate receptors are located primarily on nerve terminals and associated with primary afferent fibers comes mainly from studies of deafferentation. Deafferentation of the vagus or enucleation of the eye resulted in a marked reduction of autoradiographically identified opiate receptors in the respective primary sensory nuclei (Atweh et al., 1978). In addition, Lamotte (1976) demonstrated an approximately 50% loss of opiate specific binding sites in the dorsal horn of monkey spinal cord following transection of the dorsal roots.

A study of several narcotic agonists has revealed that several distinct clinical syndromes accompany their administration. Martin (1976) has postulated that these differences, most notably in subjective effects and types of physical dependence, are a consequence of the drug interacting with different types of the opiate receptor. He suggested that there are three types of opiate receptor located in nervous tissue and designated them  $\mu$ ,  $\kappa$ , and  $\sigma$ . Prototypic agonists for these receptors are morphine ( $\mu$ ), N-allylnormetazocine ( $\sigma$ ) and ethylketocyclazocine ( $\kappa$ ). More recently, Lord and colleagues (1977) have identified a fourth form of the receptor which has been designated  $\delta$ . These investigators found that in the guinea-pig ileum, the enkephalins are as readily antagonized by naloxone as is normorphine; however, in the mouse vas deferens they are not. About 10 times more naloxone was required to reverse the inhibition produced by enkephalins than was required to reverse the inhibition caused by normorphine. Such a difference in the  $pA_2$  values for enkephalin in the guinea-pig ileum and mouse vas deferens strongly suggests that the receptor populations in the two in vitro models

are heterogeneous and not identical. In addition, these investigators demonstrated that the inhibition of  $^3\text{H}$ -leu-enkephalin and  $^3\text{H}$ -naloxone binding in brain homogenates of guinea-pig was not equal for opiates and opiate peptides. That is, both met and leu-enkephalin displaced  $^3\text{H}$ -leu-enkephalin binding whereas morphine was a much better inhibitor of  $^3\text{H}$ -naloxone binding than of  $^3\text{H}$ -leu-enkephalin binding. Again, such findings strongly suggest the existence of a heterogeneous population of opiate receptors.



### 1.1.3. Pharmacology of Opiates

For the most part, narcotic analgesics have been classified as belonging to one of two groups. The classification is based on their acute pharmacological effects as well as their dependence producing liability. Agonists are classified as being either morphine-like or nalorphine-like (Martin et al., 1976).

#### Morphine-like agonists

The acute effects include analgesia, sedation, alteration in mood, mental clouding, euphoria, miosis, cardiorespiratory depression, nausea and constipation. The analgesic effect is relatively selective, in that sensory modalities other than pain are left intact (touch, proprioception, vibration). The patient may still recognize painful stimuli but he is not bothered or concerned over it. Morphine is more effective in relieving dull chronic pain rather than sharp intermittent pain.

Studies performed on absorption, distribution and metabolism indicate that morphine and its surrogates are not readily absorbed from the gastro-intestinal tract. It is readily accessible to the target site if given parenterally and is distributed in parenchymous tissue. The major metabolic pathway in humans is conjugation with glucuronic acid in the liver, N-demethylation and urinary excretion of free morphine subserving minor roles (Jaffe and Martin, 1975). Morphine does not accumulate in tissue, and 24 hours after the last dose, tissue concentrations are quite low. An analgesic dose of morphine (10 mg)

given subcutaneously acts within minutes and its effect typically lasts 4 to 5 hours (Jaffe and Martin, 1975).

Tolerance to and physical dependence on morphine occur with repeated use. Studies on the suppression of withdrawal in subjects physically dependent on morphine reveal that morphine at a dose which is 10-20% of a stabilization dose will markedly suppress abstinence in patients who are near maximally dependent on morphine (Martin, 1971). Methadone (Dole and Nyswander, 1965), propiram, profadol (Jasinski, Martin and Hoeldtke, 1971) and d-propoxyphene (Fraser and Isbell, 1960) will substitute for, and show cross-tolerance with, morphine in the morphine-dependent subject. Cyclazocine (Martin, Fraser, Gorodetzky and Rosenberg, 1965) and pentazocine (Jasinski, Martin and Hoeldtke, 1970) will not suppress abstinence symptoms in subjects dependent on morphine; nor will ketocyclazocine and ethylketocyclazocine suppress signs of abstinence in the morphine dependent dog (Gilbert and Martin, 1976).

The severity of the characteristic syndrome which occurs when an individual abruptly discontinues chronic use of narcotic analgesics is determined by several factors such as, specific opiate administered, daily dose, duration of use, and health and personality of the individual. When morphine is abruptly withdrawn, the ensuing abstinence syndrome is characterized by an early and protracted phase. The early phase begins manifesting symptoms at the time of the next scheduled dose and lasts 4-10 days. Early signs of opiate withdrawal are nervousness, weakness, muscle aches, lacrimation, abdominal cramps, anorexia, nausea, craving for sweets, aversion to tobacco, rhinorrhea, yawning, perspiration and restless sleep. As the syndrome approaches its peak (2-3 days in the

case of morphine and heroin), irritability, insomnia, anorexia, yawning, sneezing, weakness, depression, rise in blood pressure, pulse rate, respiratory rate and body temperature, intestinal spasm and diarrhea increase. Administration of opiate at any point during the withdrawal completely suppresses the syndrome. The early phase of abstinence in a moderately dependent individual usually subsides in 7-10 days. However, a much more prolonged return to preaddiction levels has been noted for some of the signs, especially blood pressure, pulse rate, body temperature and pupillary diameter. Between the sixth and ninth week of abstinence, physiological changes begin to emerge as a result of the discontinuation of morphine. This later phase, known as protracted abstinence, persists for more than 30 weeks. During this phase, blood pressure, pulse rate, body temperature, pupillary diameter and the sensitivity of the respiratory center to  $\text{CO}_2$  are below normal levels.

The level of tolerance and cross tolerance which develops depends upon the type of narcotic administered and its dose. There have been no thorough studies of the disappearance of tolerance following cessation of drug treatment. Most studies indicate that tolerance is attenuated a few days after cessation of drug administration when compared with the degree of tolerance at the time of cessation. Goldstein and Sheehan (1969) showed that the return to normal sensitivity to the stimulatory effect of levorphanol in mice was complete in 48 hours after withdrawal. In clinical studies narcotic addicts undergoing withdrawal reported a rapid recovery (within a few days) of the euphoric and depressant effects of narcotics (Light, 1931). However, tolerance to certain effects such as nausea and emesis, persists for months after termi-

nation of chronic morphine administration (Fraser and Isbell, 1952). Cochin and Kornetsky (1964) have reported that residual tolerance to the analgesic effects of morphine can be demonstrated in rats up to 15 months after a single injection of a large dose of morphine.

Buprenorphine is a strong analgesic which is some 25-40 times more potent than morphine in rodents. It has a rapid onset and duration of action (Cowan, Lewis and Macfarlane, 1977). In man buprenorphine is capable of producing morphine-like subjective, behavioral and analgesic effects and is 25-50 times more potent than morphine (Jasinski, Pevnick and Griffith, 1978). Buprenorphine has been also shown to possess narcotic antagonist properties (Cowan et al., 1977). In man abrupt withdrawal of chronically administered buprenorphine results in a mild abstinence syndrome with the first signs appearing 2-3 days following withdrawal. The abstinence syndrome reaches its peak intensity at 14 or 15 days after withdrawal, at which time the subjects display a drug craving behavior (Jasinski et al., 1978). Although buprenorphine is similar to morphine in certain respects (Martin et al., 1976), there are also marked differences between these drugs. First, in the buprenorphine dependent subject administration of naloxone failed to precipitate an abstinence syndrome; and second, abrupt withdrawal of buprenorphine resulted in only a very weak withdrawal syndrome (Jasinski et al., 1978). In chronic spinal dogs buprenorphine induced a state of physical dependence and also partially suppressed morphine withdrawal. Over 20 times more naloxone was required to precipitate abstinence in the buprenorphine dependent dog than would have been necessary in dogs dependent upon an equivalent amount of morphine (Martin et al., 1976). In rats,

mice and monkeys chronic administration of buprenorphine failed to produce overt signs of physical dependence (Cowan et al., 1977). No satisfactory explanation is available as to why different results were obtained with the monkey, rat and mouse as opposed to the dog and human. Perhaps, the dependence which is observed in the dog and human is minimal or clinically insignificant. It is suggested that the long duration of action and the lack of effect by naloxone in precipitating abstinence are an indication of the high affinity of the buprenorphine-receptor interaction, as binding studies indicate an extraordinarily low dissociation rate constant (Rance and Dickens, 1978).

#### Nalorphine-like agonists

Cyclazocine is a very potent and long acting narcotic antagonist, but when given alone has narcotic analgesic properties. In man cyclazocine (0.25 - 2.0 mg) produces a state of analgesia comparable to that seen with morphine 20 mg (Lasagna, DeKornfeld and Pearson, 1964). Subjective effects of cyclazocine closely resemble those seen with nalorphine and consist of feelings of apathy, sleepiness, tiredness, irritability, racing thoughts, delusions, and hallucinations. The majority of subjects do not either like or dislike the drug (Fraser and Rosenberg, 1966). In the chronic spinal dog cyclazocine produces nalorphine-like effects in single doses and physical dependence with chronic administration (Gilbert and Martin, 1976).

Chronic administration of cyclazocine to man induces a state of physical dependence. Such subjects show tolerance to all major

pharmacological effects of cyclazocine as well as cross-tolerance to nalorphine. Abrupt withdrawal or administration of naloxone results in an abstinence syndrome which closely resembles that following nalorphine but which is clinically different to the one seen in morphine dependent subjects (Jasinski, Martin and Sapira, 1968; Martin et al., 1965). After subjects have been stabilized on parenteral doses of cyclazocine for several weeks, abrupt withdrawal results in an abstinence syndrome which does not manifest itself until the fourth day and which does not become maximal until the seventh day (Martin et al., 1965). Signs of abstinence consist of mydriasis, increased pulse rate and body temperature, decreased body weight, lacrimation and diarrhea. Certain signs persist for periods as long as 6 weeks following withdrawal (Martin, Fraser, Gorodetzky and Mc Clane, 1965). The syndrome has been characterized as being slow in onset and mild. No compulsion to take the drug is apparent upon withdrawal as is common with morphine addiction. In short, although an abstinence syndrome ensues upon abrupt withdrawal of cyclazocine, the withdrawal syndrome does not produce a great desire to continue taking the drug and, therefore, liability for abuse of cyclazocine seems slight. Nalorphine and ketocyclazocine both suppress signs of cyclazocine withdrawal in man and dog (Fraser and Rosenberg, 1966; Gilbert and Martin, 1976).

Ketocyclazocine is a narcotic analgesic of the nalorphine type. It has an analgesic activity equivalent to cyclazocine in the rat abdominal constriction test (Michne, Pierson and Albertson, 1974). Chronic administration leads to the development of tolerance and physical dependence. Ketocyclazocine does not suppress abstinence symptoms in the

morphine dependent rat (Michne et al., 1974), monkey (Swain and Seevers, 1974), or dog (Gilbert and Martin, 1976). Characteristics of keto-cyclazocine withdrawal closely resemble those of cyclazocine and, therefore, differ from those of morphine.

#### 1.1.4. Mechanisms and Theories of Opiate Tolerance and Dependence

The question of whether opiate tolerance and dependence exists as two separate but closely related phenomena, or as quite inseparable phenomena with a common underlying mechanism remains unresolved. Evidence in support of the hypothesis that the two phenomena are inextricably linked includes; 1) the finding that physical dependence seems to develop in parallel with analgesic tolerance (Way, Loh and Shen, 1969), 2) the greater the level of tolerance attained the harsher the withdrawal syndrome (Martin, 1971), and 3) all of the narcotics known to man cause both phenomena (Martin, 1971).

Several theories on the mechanism of tolerance have been proposed, but none appears to have been defined precisely and to have established its role in the development of tolerance and dependence.

#### Counter-Adaptive Theory of Himmelsbach

One of the earliest theories of opiate tolerance and dependence was proposed by Himmelsbach (1943). In this theory, the effect of opiates on hypothalamic centers is to disturb homeostasis. This leads to physiologic adaptations which oppose the acute actions of opiates. That is, repeated exposure to the narcotics leads to a new equilibrium in which the narcotic drug is an essential component. When the narcotic is withdrawn, it disrupts this new state of equilibrium; therefore, the physiologic adaptations are no longer balanced by the opiate, and a withdrawal syndrome is observed in which the clinical manifestations are



usually in directions opposite to those following the acute administration of opiates.

### Altered Disposition

A second theory of the mechanism of tolerance and dependence was based on the differences in biologic disposition between tolerant and non-tolerant subjects (Light, 1931; Axelrod, 1956; Hug, 1972). However, thorough investigations have revealed no differences in the excretion of either free or bound morphine in tolerant and non-tolerant dogs (Cochin, Haggart, Woods and Seevers, 1954) or in the distribution and fate of morphine in tolerant dogs, rats (Woods, 1954) or monkeys (Mellett and Woods, 1956).

It is generally accepted that differences in absorption, distribution, metabolism and excretion of narcotic analgesics cannot possibly account for the differences in pharmacological responses observed between tolerant and non-tolerant subjects.

### Role of the Opiate Receptor

Several studies have demonstrated that naloxone, given before or concurrently with morphine, to rats or mice, blocked the development of tolerance to and dependence on morphine (Mushlin, Griffel and Cochin, 1975; Yano et al., 1979). Such findings lead investigators to conclude that the opiate receptor was somehow necessary for the induction of tolerance and dependence. Studies were made to determine whether changes in receptor number or affinity paralleled the development of tolerance and dependence.

Pert and coworkers (1973) were the first to demonstrate an in-

crease in stereospecific binding of  $^3\text{H}$ -dihydromorphine in mice implanted with morphine pellets. Similarly, Frederickson et al. (1974) reported an increase in the number of opiate binding sites in rats rendered tolerant and dependent on morphine. Conversely, several investigators have reported a decrease in the number of opiate specific binding sites with the development of opiate tolerance and dependence. Davis and coworkers (1975) found that with chronic morphine therapy a decrease in receptor affinity to morphine occurred in brainstem slices; however, these same investigators found no difference in binding properties when binding was measured in brainstem homogenates. In contrast to the aforementioned studies, the majority of investigations suggest no change in either the affinity or number of opiate receptors. Klee and Streaty (1974) were the first to show no change in the stereospecific binding for either  $^3\text{H}$ -dihydromorphine or  $^3\text{H}$ -naloxone in brains from naive and opiate tolerant rats. Similar findings were reported by Bonnet (1976). The reasons for these disparities in results remain unknown; they may be related to differences in experimental technique, approach and methods used by the various investigators.

It is difficult to assess the significance of such experiments because the binding properties which are recorded are those of a tissue which is undergoing withdrawal from opiate rather than a tissue which continues to be tolerant and dependent on opiate. This is because the opiate must be withdrawn from the tissue for the binding studies to be performed. This procedure of washing the tissue (in vitro) induces a state of opiate withdrawal and perhaps an altered (conformational) state of the receptor. Thus, such findings may not be a reliable index of opiate

receptor properties during the phenomena of tolerance and dependence. In general, it is fairly well accepted that changes in the opiate receptor, if in fact they do occur, cannot adequately account for the changes observed during opiate tolerance and dependence. Therefore, it appears likely that this phenomenon is somehow dependent upon events which follow receptor occupation.

### Immune Mechanisms

A number of observations have suggested that immunoreactions may be involved in opiate tolerance and dependence (Cochin, 1970). In rabbits, chronic morphine administration resulted in an increased capacity of serum proteins to bind morphine by as much as 100 fold (Herndon, Baeder and Ringle, 1974) and there is evidence that serum proteins of heroin addicts have a greater affinity for heroin than serum proteins from drug naive individuals (Ryan, Parker and Williams, 1972). Immunization of mice with a morphine immunogen (3-carboxymethylmorphine-BSA) resulted in the formation of an antibody to morphine. In these mice, the analgesia produced by a single dose of morphine was significantly less than the analgesia produced by morphine in the adjuvant treated animals (Berkowitz and Spector, 1972). This finding suggests that tolerance to the analgesic effect of morphine was accelerated by the formation of antibody. A problem with this theory is that it does not adequately explain cross-tolerance among structurally unrelated narcotics. For instance, antibodies to morphine do not readily cross react with methadone (Wainer, Wung, Connors and Rothbert, 1979), yet methadone is clearly less effective in morphine tolerant subjects (Dole and Nyswander, 1965).

## Cyclic Nucleotides

Administration of a phosphodiesterase inhibitor induces a syndrome in rats which is indistinguishable from the syndrome seen upon cessation of chronic morphine therapy (Francis, Roy and Collier, 1975). This behavioral pattern elicited by the phosphodiesterase inhibitor is known as the quasi-morphine abstinence syndrome (QMAS). A fair correlation has been found between the ability of various agents to inhibit phosphodiesterase and the intensity of the withdrawal syndrome (Francis, Cuthbert, Saeed, Butt and Collier, 1978). The quasi-morphine abstinence syndrome is potentiated by narcotic antagonists and suppressed by narcotic agonists (Collier, Francis, Henderson and Schneider, 1974). In addition, phosphodiesterase inhibitors or cyclic-AMP alone will intensify the naloxone precipitated withdrawal syndrome in morphine dependent rats (Francis, Roy and Collier, 1975; Collier and Francis, 1975). Consistent with the premise that the cyclic nucleotides play a prominent role in opiate tolerance and dependence is the finding by Ho and colleagues (1973b) that administration of cyclic-AMP to mice enhanced the development of opiate tolerance and dependence. On the other hand, chronic administration of  $\Delta^9$ -tetrahydrocannabinol (Kaymakcalan, Hakkiayhan and Cankattulany, 1977) or a single microinjection of histamine in the hippocampus of the rat (Glick and Crane, 1978) and probably other procedures can induce an opiate-like abstinence syndrome. These compounds are neither opiate-like nor do they inhibit phosphodiesterase.

Studies of neuroblastoma-glioma hybrid cells (NG108-15) have demonstrated that after an initial decrease in cyclic-AMP levels by

opiates there is a compensatory increase to control levels. That is, cyclic-AMP levels return to normal values while the opiate is present (acquisition of tolerance). The hybrid cells also acquire a dependence on opiates, in the sense abrupt withdrawal of the opiate or addition of naloxone to the tissue results in an increase in cyclic-AMP levels well in excess of the original level (Sharma, Klee and Nirenberg, 1975), Tolerance and dependence in NG108-15 cells are also elicited by opioid peptides such as methionine-enkephalin (Klee, 1978).

The experiments described have led to the concept of dual regulation of adenylate cyclase as a biochemical model for the acute and chronic actions of opiates. The inference from these studies is that the compensatory increase in cyclic-AMP levels is responsible for symptoms associated with opiate withdrawal (see section 1.3).

#### Inhibitors of Tolerance and Dependence

The early findings that calcium ion antagonized the analgesic effect of opiates (Kakunaga, Kaneto and Hano, 1966) prompted further studies on the role of calcium in opiate tolerance and dependence. Kaneto (1971) reported that mice given repeated intracisternal injections of morphine plus calcium were less tolerant to the analgesic effect of morphine than were the group receiving morphine alone. Calcium was also found to inhibit certain aspects of the naloxone precipitated withdrawal in morphine dependent mice (Harris, Bhargava, Loh and Way, 1974). The mechanism by which calcium inhibits the development of tolerance and the symptoms of withdrawal is not known. However, it may be linked to its involvement with neurotransmitter release and a possible interaction between calcium and morphine with respect to release because opiates in-

hibit transmitter release at various sites in the nervous system (Paton, 1957; Sanfacon and Lambrequé, 1977; Henderson and Hughes, 1976).

Protein synthesis inhibitors have been shown to attenuate morphine tolerance and dependence. Tolerance to the analgesic effect of morphine was reduced or prevented by several drug treatments which inhibited protein synthesis. That the effect of these agents are not due to lethargy or debilitation of the animal or alteration in metabolic disposition was shown by Cox and Osman (1970) and Feinberg and Cochin (1977). The inhibitors studied were cycloheximide, 5-fluorouracil, 6-mercaptopurine, actinomycin D and puromycin. The study by Cox and Osman (1970) was designed to reduce the complications due to systemic toxicity of the various agents. Infusion of small doses of protein synthesis inhibitors intracerebroventricularly or intravenously inhibited the development of acute tolerance to morphine produced during a 3 to 7 hour continuous infusion. The results support the hypothesis that protein synthesis is an integral function in the development of tolerance to narcotics in vivo.

Neurohypophyseal peptides, as well as certain of their derivatives, have been recently shown to alter the development of opiate tolerance and dependence in rodents (Walter et al., 1978; 1979). The mechanism of action by which these peptides exert their effect remains obscure.

#### Enkephalins and $\beta$ -endorphin

The involvement of enkephalins or  $\beta$ -endorphin in the mechanism underlying opiate tolerance and dependence is not clearly understood.

It is known that cross-tolerance between morphine and enkephalins occurs in the guinea-pig ileum and mouse vas deferens (Waterfield, Hughes and Kosterlitz, 1976) and with respect to their analgesic effect in the rat (Pert, 1976). Kosterlitz and Hughes (1975) have suggested that the development of tolerance to morphine may be associated with a decrease in the levels and synthesis of enkephalin. It was further suggested that a deficiency of an endorphinergic system could explain the protracted phase of the abstinence syndrome (Goldstein, 1976) and the high rate of recidivism after abstinence in heroin addicts (Przewlocki, Holt, Duka, Leber, Gramsch, Haarman and Herz, 1979). However, no change in either  $\beta$ -endorphin or enkephalin levels were detected in rat brains from rats subjected to acute or chronic morphine treatment over a maximum period of 10 days (Childers et al., 1977; Fratta et al., 1977; Wesche et al., 1977), although a decrease in the  $\beta$ -endorphin levels was observed (in rat brain) when morphine therapy was extended for periods of 30 days (Przewlocki et al., 1979). Therefore, a relatively long exposure to morphine appears to be necessary to decrease  $\beta$ -endorphin or enkephalin levels in rat brain, and to effect the development of opiate dependence and characteristics of withdrawal.

## 1.2. SINGLE NEURON STUDIES

### 1.2.1. Acute Effects of Opiates

Early electrophysiological studies on the action of opiates were limited to those brain areas associated with pain and its transmission. The discovery and localization of opiate receptors in discrete brain regions (Pert and Snyder, 1973) redirected the studies of single neurons toward areas enriched with these receptors. Three criteria must be fulfilled before an opiate effect may be considered receptor mediated. First, the effect should be observed at a concentration of opiate which is pharmacologically relevant. Second, the effect should be stereospecific; that is, mimicked only by the (-) isomer of enantiomeric opiates. Third, naloxone should antagonize the effect. The use of naloxone reversibility as the sole criteria for opiate specificity should be viewed with caution since naloxone has been shown to reverse the inhibitory effect of  $\gamma$ -aminobutyric acid (GABA) on the firing rate of neurons located within the olfactory tubercle (Dingledine, Iversen and Breuker, 1978), the fatigue of peristalsis induced by adenine nucleotides, haloperidol and xylocaine in the guinea-pig ileum (Van Nueten, Jansen and Fontaine, 1976a; 1976b) and the antinociception produced by phenoxybenzamine in mice (Spiehlner and Paalzow, 1979). However, it remains to be determined whether these effects are mediated through the release of enkephalin.

There is generally a good correlation between the density of opiate binding sites and the number of cells which are responsive to



opiates and opioid peptides (Nicol, Siggins, Ling, Bloom and Guillemin, 1977). Opiates administered either systemically or by iontophoresis inhibit the spontaneous firing of neurons in the rat sensorimotor cortex (Sato, Zieglgänsberger, Fries and Herz, 1974), hypothalamus (Eidelberg and Bond, 1972), medial thalamus (Linseman, 1978), parafascicular nucleus (McClung and Dafny, 1978), locus coeruleus (Korf, Bunney and Aghajanian, 1974; Bird and Kuhar, 1977), and caudate nucleus (Dafny et al., 1979).

A number of studies have shown that the excitatory response of single neurons to the stimulation of their peripheral receptive fields are inhibited by opiates. In the rat, morphine inhibits the neuronal activity evoked by noxious stimulation in the mesencephalic reticular formation (Haigler, 1976), lamina V cells of the spinal cord (Calvillo et al., 1974; Duggan et al., 1977b), thalamus (Hill and Pepper, 1978) and locus coeruleus (Korf et al., 1974). One area which has been extensively studied with respect to selective depression of nociceptive activity by narcotic analgesics is the dorsal horn of the spinal cord of cats (Kitahata et al., 1974; Duggan et al., 1977b). Iontophoretic application of morphine into the substantia gelatinosa or systemic administration (1-2 mg/kg i.v.) produced a selective depression of dorsal horn cell responses to noxious stimulation, but did not affect responses to non-noxious stimuli. These effects were reversed by low concentrations of naloxone (0.1 mg/kg i.v.) (Duggan et al., 1976; 1977b).

Whereas opiates usually inhibit neuronal activity, there are at least two areas in the CNS where the predominant effect of opiates and opioid peptides is neuronal excitation. These are the Renshaw cells

of cats (Davies, 1976; Davies and Dray, 1978) and the hippocampal pyramidal cells of rats (Nicolle et al., 1977). However, we may not deal here with a direct increase in neuronal activity, as Zieglgänsberger and colleagues (1978) have provided evidence that the opiate induced excitation of hippocampal pyramidal cells is due to inhibition of neighboring tonically active inhibitory neurons. It is possible that the excitation of Renshaw cells may have a similar explanation.

The present review of the electrophysiological study of opiates in vivo is incomplete. The reader is referred to reviews by Bradley (1978) and North (1979a) for a complete and detailed description of the action of opiates in vivo.

#### In Vitro Studies

Sato and colleagues (1973) were the first to demonstrate an effect of morphine on myenteric neurons in vitro. In this study, morphine (approximately 8.5  $\mu$ M) inhibited the spontaneous activity of single myenteric neurons. These same investigators later demonstrated that morphine (approximately 3  $\mu$ M) inhibited neurons whether they were firing spontaneously or induced to fire by 5-hydroxytryptamine (5-HT) or caerulein (Sato, Takayanagi and Takagi, 1974). The concentrations of morphine used in these studies were quite high, considerably greater than the concentration necessary to inhibit the electrically induced contraction of the longitudinal muscle myenteric plexus preparation of the guinea-pig ileum (ID<sub>50</sub> 85 nM) (Goldstein and Schulz, 1973). Subsequent studies by Dingledine and Goldstein (1975; 1976) have demonstrated that the inhibitory effect of morphine on myenteric neurons could be reproduced using a concentration of morphine comparable to those used in the

twitch studies. The inhibition of firing was stereospecific, that is it was mimicked by levorphanol but not dextrorphan, and was antagonized by low concentrations of naloxone (Dingledine, Goldstein and Kendig, 1974; Dingledine and Goldstein, 1975). Their findings confirm and extend those by Sato's group. In addition, morphine was shown to be effective under conditions of synaptic transmission blockade (Dingledine and Goldstein, 1976; North and Williams, 1977). The inference from these studies as well as those by Sato in which morphine depressed the excitations induced by 5-HT, caerulein and nicotine was that morphine probably acts directly on the myenteric neuron to reduce excitability. Similar results were obtained with the endorphins (North and Williams, 1976; Williams and North, 1979a). The mechanism of action of opiates on single myenteric neurons is discussed in detail below.

Studies of dorsal root ganglia and spinal cords dissociated from each other and allowed to make connections in culture have demonstrated a possible presynaptic site of opiate action (Crain, Peterson, Crain and Simon, 1977; MacDonald and Nelson, 1978). In the study by Crain et al. (1977), electrical stimulation of the attached dorsal root ganglion resulted in a complex negative slow wave potential which was selectively depressed by opiates. The effect was seen with low concentrations of morphine, etorphine and levorphanol, and was antagonized by naloxone. Dextrorphan had no effect. Naloxone alone in many cases selectively increased the amplitude and duration of the sensory evoked potential in the dorsal horn. The inference was that these sensory networks develop localized opioid inhibitory control systems mediated by the endorphins.

In the other study, intracellular recordings were made from spi-

nal neurons. Stimulation of the dorsal root ganglion gave rise to an excitatory postsynaptic potential (e.p.s.p.) in spinal neurons. The e.p.s.p. was depressed by iontophoretically applied etorphine. Since etorphine did not have any postsynaptic effect of its own, the authors concluded that etorphine exerted its effects by inhibiting the release of transmitter from the primary afferent terminals (MacDonald and Nelson, 1978).

There are at least two viewpoints as to the underlying mechanism of opiate action in single mammalian neurons. One view holds that morphine acts postsynaptically to block the inward sodium current induced by glutamate or possibly other excitatory agents at the subsynaptic membrane. Intracellular recordings of spinal motoneurons in vivo revealed no significant changes in the membrane potential or input resistance to iontophoretically applied morphine but it was found that morphine reduced the rate of rise of the e.p.s.p. and the potential evoked by iontophoretic application of glutamate (Zieglgänsberger and Bayerl, 1976). A second view proposes a membrane hyperpolarizing effect of morphine as the underlying mechanism of action. A significant proportion of myenteric neurons recorded in vitro were hyperpolarized by opiates and opiate peptides (North and Tonini, 1977; North, Katayama and Williams, 1979). It has been proposed that such a hyperpolarizing effect may explain not only the inhibition of neuronal firing but also the inhibition of acetylcholine release evoked by field stimulation. Furthermore, it appears likely that the site of opiate action in the myenteric plexus is non-somatic; that is, morphine acts by hyperpolarizing the nerve processes. Morphine showed no consistent effects on the amplitude of the excitatory postsynaptic potential (North, 1979b).

### 1.2.2. Chronic Effects of Opiates

When rats were chronically exposed to morphine by means of subcutaneous pellet implantation or by multiple subcutaneous (or intraperitoneal) injections, no change in the pattern or frequency of the spontaneous neural firing was reported for the opiate treated animals as compared with drug naive animals (Sato, Zieglgänsberger and Herz, 1976). Furthermore, tolerance to the usual depressant effect of morphine on spontaneous activity or activity evoked by acetylcholine or glutamate was demonstrated on neurons in the rat sensorimotor cortex (Sato et al., 1974), hypothalamus (Eidelberg and Bond, 1972), parafascicular nucleus (McClung and Dafny, 1978) and locus coeruleus (Aghajanian, 1978) (Table 1.). Similarly, tolerance to the inhibitory effect of morphine on single neuron activity has been demonstrated in the medial thalamus, periaqueductal grey, caudate nucleus, hippocampus and nucleus accumbens of rabbits (Peters and Klem, 1977).

Naloxone was without effect on the firing rate of neurons in drug naive animals, whereas it induced a marked excitation of neurons in opiate dependent animals (Table 1.). In opiate dependent rats, those brain sites in which naloxone increased neuronal activity included the sensorimotor cortex (Sato et al., 1975), hypothalamus (Eidelberg and Bond, 1972), medial thalamus (Frederickson, Morris and Hewes, 1975) and the locus coeruleus (Aghajanian, 1978). This increase in neuronal activity induced by naloxone has been referred to by some investigators as a manifestation of opiate dependence. Similar results have been reported

Table 1. The action of morphine and naloxone on the firing rate of neurons of drug naive and opiate pretreated animals.

Abbreviations: morph morphine  
nalox naloxone  
ne no effect  
↑ firing rate increased  
↓ firing rate decreased  
-- not tested

Animal	Region	Predominant Response				Reference
		Naive		Tolerant / Dependent		
		morph	nalox	morph	naloxone	
Rat	Sensorimotor cortex	↓	ne	↑	↑	Sato et al., 1974
Rat	Hypothalamus	↓	ne	↑	↑	Eidelberg and Bond, 1972
Rat	Medial thalamus	↓	ne	--	↑	Frederickson et al., 1975 Linseman, 1978
Rat	Parafascicular nucleus	↓	--	↑	--	McClung and Dafny, 1978
Guinea pig	Myenteric ganglion	↓	ne	ne	↑	North and Williams, 1977; North and Zieglgänsberger, 1978
Rat	Locus coeruleus	↓	ne	↓	↑	Aghajanian, 1978
Rat	Cerebellum (Purkinje cells)	ne	ne	ne	ne	Aghajanian, 1978
Rat	Mesencephalic nucleus of the 5th nerve	ne	ne	ne	ne	Aghajanian, 1978
Rat	Reticular formation	ne	ne	ne	ne	Aghajanian, 1978
Rat	Spinal interneuron	ne	ne	ne	ne	Davies, 1976
Rat	Brainstem	↑	ne	↑	↑	Bramwell and Bradley, 1978

for peripheral mammalian neurons studied in vitro. Extracellular recordings from myenteric neurons from guinea-pigs pretreated with morphine demonstrated tolerance to the usual inhibitory effect of morphine. A naloxone-induced excitation was also observed (North and Zieglgänsberger, 1978).

Changes in neuronal sensitivity occur concomitantly with the development of tolerance. The sensitivity to excitations by glutamate of striatal neurons (of drug naive rats) increased during and after a prolonged microelectrophoretic application of met-enkephalin: at the same time, the inhibitory effect of met-enkephalin progressively diminished. This tachyphylaxis to the inhibitory effect of met-enkephalin occurring concurrently with an increased sensitivity to glutamate may or may not be related to the tolerance described (Fry, Zieglgänsberger and Herz, 1978). The mechanism underlying the changes in neuronal sensitivity has not been thoroughly investigated; however, one hypothesis suggests a membrane depolarization as the basis for the effect (Johnson, Westfall, Howard and Fleming, 1978). In support of this view, is the membrane depolarization recorded when naloxone is administered to tissue (myenteric neurons) which has been pretreated (20 h) with a morphine solution (Johnson and North, 1980). It is believed that tissue exposed in this manner is tolerant to and dependent on morphine (Hammond et al., 1976).



### 1.3. THE INTERACTION OF CYCLIC-AMP AND RELATED AGENTS WITH OPIATES

#### 1.3.1. Antinociception

Recent studies on the biochemical basis of opiate analgesia have implicated the cyclic nucleotide system(s). It has been demonstrated that cyclic adenosine-3',5'-monophosphate (cyclic-AMP), dibutyryl cyclic adenosine-3',5'-monophosphate (dbcyclic-AMP), or theophylline administered intracerebrally or intravenously antagonizes the antinociception produced by morphine in mice (Ho, Loh and Way, 1973a). Similarly, prostaglandin  $E_1$  ( $PGE_1$ ) administered intraventricularly reversed the antinociception produced by morphine in the rat (Feri, Santagostino, Braga and Galatulas, 1974). This effect of  $PGE_1$ , which is to reverse the antinociception produced by morphine, may be due to its ability to elevate cyclic-AMP levels (Collier and Roy, 1974; Welman and Schawabe, 1973). Nevertheless, the mechanism whereby these compounds reverse the analgesic effect of morphine has not been completely elucidated. It may be that they are indeed exerting their effect by directly altering intracellular levels of cyclic nucleotides, or perhaps by affecting neurotransmitter release (Nathanson, 1977; Schulz and Cartwright, 1976) or mobilizing  $Ca^{++}$  stores (Ramwell and Shaw, 1970; Hayashi, Mori, Yamada and Kunimoto, 1978). The acute actions of opiates have been shown to be related to each of these factors.

### 1.3.2. Studies on Brain Homogenates and Neuroblastoma-Glioma

#### Hybrid Cells

Studies by Collier and Roy (1974) provided the earliest biochemical evidence linking the opiates to adenylate cyclase activity. These workers found that the conversion of  $^3\text{H}$ -ATP to  $^3\text{H}$ -cyclic-AMP was stimulated approximately two fold by prostaglandin  $\text{E}_1$ . Morphine inhibited this stimulation but was without effect on the basal production of cyclic-AMP. The inhibitory effect was dose related, mimicked by heroin but not dextrorphan, and reversed by naloxone. Similar findings in homogenates of monkey amygdala (Wilkening, Mishra and Makman, 1976), and in brain slice preparations (Minneman and Iverson, 1977) have confirmed the results by Collier and Roy (1974), although a number of other laboratories have been unsuccessful in their attempts to do so (Katz and Catravas, 1977; Van Inwegen, Strada and Robison, 1975). Several explanations have been offered to account for these discrepancies, such as regional heterogeneity of cell types in brain homogenates, lability of brain enzymes under experimental conditions, inability to control factors which are known to affect adenylate cyclase activity such as ions and neurotransmitters, and the possible variable effects of morphine on cyclic-AMP levels in different brain regions (Bonnet, 1975).

Studies on neuroblastoma-glioma hybrid cell (NG108-15) have revealed an inhibitory action of morphine on basal and  $\text{PGE}_1$  stimulated cyclic-AMP levels. The parent cell lines, neuroblastoma N18TG-2 and glioma C6BU-1, are not significantly affected by opiates and this agrees

with the finding by several investigators that they possess few if any opiate binding sites (Klee, Sharma and Nirenberg, 1975). The hybrid cells on the other hand, possess opiate receptors (Klee, Sharma and Nirenberg, 1975; Chang, Cooper, Hazum and Cuatrecasas, 1979). Morphine inhibits basal,  $PGE_1$ , and adenosine stimulated cyclic-AMP formation in these cells. These effects are stereospecific and naloxone reversible (Sharma, Klee and Nirenberg, 1975). In the continued presence of morphine (several hours), the initial inhibition of adenylate cyclase in these hybrid cells is followed by an increase in enzyme activity. After a 12 hour exposure to morphine, adenylate cyclase activity has returned to normal and the cells could be said to be tolerant. Withdrawal of the morphine or addition of naloxone leads to a rapid increase in cyclic-AMP formation which exceeds the original level (Sharma, Klee and Nirenberg, 1975); this has been interpreted as a sign of dependence. Thus, tolerance to and dependence on morphine have been induced in neuroblastoma-glioma hybrid cells in vitro. The effects were only partially blocked by the protein synthesis inhibitor cycloheximide, implying perhaps, only a limited role for protein synthesis in the underlying mechanism (Sharma, Klee and Nirenberg, 1977).

### 1.3.3. Myenteric Plexus

Adenosine and the adenine nucleotides such as adenosine triphosphate (ATP), cyclic adenosine 3'-5'-monophosphate and dibutyryl cyclic adenosine 3'-5'-monophosphate depress the nerve mediated contractions of the longitudinal muscle of the guinea-pig ileum by inhibiting the release of acetylcholine (Takagi and Takayangi, 1972; Sawynok and Jhamandas, 1976; Hayashi, Mori, Yamada and Kunitomo, 1978). Several xantine derivatives including theophylline have been reported to antagonize this inhibitory effect (Hayashi et al., 1978; Sawynok and Jhamandas, 1976). In addition, theophylline has been reported to reverse the inhibition of the contractile response by morphine but not by tetrodotoxin, adrenalin and strychnine (Hayashi et al., 1978; Hammond, Schneider and Collier, 1976). The mechanism whereby theophylline reverses the inhibitory effects of both morphine and adenosine is not clearly understood. It appears unlikely that a common mechanism mediates the reversal of the effects by both drugs for the following reasons. First, there is evidence that the adenine nucleotides mediate their depressant effect by activation of an extracellular adenosine receptor and that theophylline is a competitive antagonist at this receptor (Hayashi et al., 1978). Two, the low concentration of theophylline necessary to antagonize the adenosine response does not itself alter the size of the nerve mediated contraction, whereas the higher concentration of theophylline necessary to reverse the morphine depression, itself, increases the nerve-mediated contraction.

It may be that an alteration in the intracellular calcium dis-

position is involved in the underlying mechanism of action since the antagonism of morphine is seen only with those phosphodiesterase inhibitors which mobilize calcium stores. For example, the phosphodiesterase inhibitors theophylline and xanthine antagonize morphine's inhibitory action on the twitch response of the guinea-pig ileum, whereas Ro 20-1724, a non-xanthine phosphodiesterase inhibitor, does not. Theophylline and xanthines mobilize calcium stores whereas Ro 20174 does not (Hayashi et al., 1978; Gintzler and Mussachio, 1975). Therefore, it seems likely that it is the mobilization of the neuronal calcium stores and not phosphodiesterase inhibition which is the mechanism by which theophylline reverses opiate inhibition.

#### 1.4. THE MYENTERIC PLEXUS

##### 1.4.1. Anatomy

The "enteric nervous system" consists of two major ganglionated plexuses of considerable complexity (Gabella, 1976). One plexus is situated between the longitudinal and circular muscle layers and is known as the myenteric or Auerbach's plexus. The second plexus is located in the submucosa and is the submucous or Meissner's plexus. They extend uninterrupted from the lower part of the esophagus to the anus and send nerve fibers to the musculature, blood vessels and mucosa. Single ganglia within the plexus contain between a few to several hundred neurons. The number of neurons depends upon the species studied as well as the segment of the alimentary canal under investigation (Gabella, 1976). Glial cells are numerous and in the guinea-pig outnumber neurons by a ratio of 3:1 (Gabella, 1972). The entire surface of the ganglia is encapsulated by a very thin sheet of collagen (Taxi, 1965).

In the myenteric plexus of the guinea-pig ileum, the ganglia are arranged in a regularly spaced manner with many interconnecting strands located between them. The number of neurons within each ganglia varies between 20 to 200. The neurons range in size from 15 to 45  $\mu\text{m}$  in length. With respect to histological organization, the presence of a dense neuropil, absence of an extracellular space, compactness of structure, arrangement of blood vessels and connective tissue make the cells of the myenteric plexus more like CNS tissue than autonomic ganglia (Gabella, 1972).

Studies of myenteric neurons by light microscopists have demonstrated the presence of three types of neurons. This classification was based primarily on morphology (see Schofield, 1968). However, classification of myenteric neurons on the basis of ultrastructural characteristics has revealed the presence of nine distinct types of ganglion cells (Cook and Burnstock, 1976). The classification is based on neuronal size, distribution of organelles and their location and relationship to satellite cells. Similarly, eight different types of axon profiles were identified according to vesicular size, shape and content (Cook and Burnstock, 1976). Still other studies have utilized intracellular staining techniques to identify and describe myenteric neurons (Nishi and North, 1973a). These studies have demonstrated that most neurons are multipolar and have from one to seven processes. The processes usually arise from the poles of the oval soma and are varicose; however, some are smooth.

The enteric nervous system of vertebrates contains a multiplicity of neurotransmitters. Cholinergic innervation of the gastrointestinal tract of the cat, guinea-pig and monkey has been studied using the histochemical method for acetylcholinesterase or choline acetylase (Jacobowitz, 1965; Feldberg and Lin, 1950). From these and other studies (Koelle, Koelle and Friedenwald, 1950) it was established that the intramural plexuses consist of cholinergic neurons whose postganglionic fibers innervate the intestinal musculature. Adrenergic cell bodies do not exist in the myenteric plexus of the guinea-pig ileum but there is an adrenergic innervation from the prevertebral ganglia (Gabella, 1976). Norberg (1964) and Jacobowitz (1965) using fluorescence microscopy

demonstrated the presence of numerous adrenergic nerve fibers terminating in the myenteric plexus. Denervation of the sympathetic nerves led to a marked reduction of noradrenaline fluorescence in the gut (Furness, 1969). The presence of 5-HT in nerve terminals and its uptake and synthesis in cultured myenteric neurons suggests that 5-HT may function as a neurotransmitter in the gut (Dreyfus, Sherman and Gershon, 1977; Dreyfus, Bornstein and Gershon, 1977). However, these results await further confirmation. Finally, immunohistochemical studies have demonstrated the localization of enkephalin (Elde, Hokfelt, Johansson and Terenius, 1976), substance P (Nilsson, Larsson, Hakansson, Brodin, Pernow and Sundler, 1975), vasoactive intestinal polypeptide (VIP) (Larsson et al., 1976) and somatostatin (Costa, Patel, Furness and Arimura, 1977) in nerve fibers and/or cell bodies in the myenteric plexus of the guinea-pig.

Electrophysiological studies have demonstrated a clear functional role for acetylcholine in the myenteric plexus (see below) (Nishi and North, 1973a). The action of noradrenaline in the myenteric plexus is to inhibit the presynaptic release of neurotransmitter (Nishi and North, 1973b; Hirst and McKirdy, 1974); and the functional role of substance P (Katayama and North, 1978) or 5-HT (Wood and Mayer, 1978) may deal with slow excitatory synaptic transmission. Extracellular recordings of myenteric neurons in vitro have demonstrated excitatory effects of substance P (Williams and North, 1978a) and VIP (Williams and North, 1979b), and inhibitory effects of enkephalin (North and Williams, 1976) and somatostatin (Williams and North, 1978b). These are discussed in greater detail in sections 1.4.2. and 1.4.3.



#### 1.4.2. Electrophysiology

Extracellular Recordings. The first to report on extracellular recorded action potentials from single myenteric neurons in vitro was Yokayama in 1966. Subsequent studies by Wood (1970) and Ohkawa (1972) further demonstrated three types of myenteric neurons. This classification was based on the pattern and frequency of spike discharge. These studies were performed on myenteric neurons from cat jejunum and employed similar experimental methods. The three types of neurons described by these investigators were 1) single spike 2) burst and 3) mechanosensitive. Single spike type cells continuously fired action potentials at low frequencies (0.1 - 1 Hz) and with no consistent pattern of discharge. Burst type cells fired action potentials at very high frequencies (2 - 34 Hz) but only intermittently (intervals between bursts of 0.5 - 40 sec). Mechanosensitive cells responded to mechanical distortion with an increased rate of discharge; for example, pressure applied to the ganglion by the recording electrode served to activate these neurons. Several subclasses for each of the types have been reported by Wood (1970).

On the other hand, electrophysiological studies by Dingledine (1974; 1975) and Sato (1973) demonstrated only two types of myenteric neurons in the guinea-pig. They classified the cells as being of the single spike or burst type. The majority of neurons recorded were of the single spike type whereas Wood (1970) and Ohkawa (1972) found that the majority of cells were of the burst type. The difference in the

number of cell types and the type most frequently recorded can be explained as due to a difference in the experimental techniques used by the two groups. For instance, the studies by Wood (1970) and Ohkawa (1972) utilized metal electrodes for recording while those of Dingle-dine and Sato utilized glass suction electrodes. It is possible that a particular type of electrode may preferentially select a distinct neuronal type.

Typically, action potentials of myenteric neurons were biphasic, ranging in amplitude from 50 to 300  $\mu$ V and in duration from 2 - 4 ms. The spikes were abolished by tetrodotoxin and local anesthetics (Wood, 1970; Sato et al., 1973; Dingledine et al., 1974; North and Williams, 1977). Mechanosensitive and burst type neurons were relatively insensitive to most drugs, whereas single spike units were sensitive to a variety of agents. Single spike neurons were excited by 5-HT, acetylcholine, nicotine, pentagastrin, caerulein (Sato et al., 1973), substance P (Williams and North, 1978a), neurotensin (Williams, Katayama and North, 1979), and VIP (Williams and North, 1979b). The firing of these neurons was inhibited by morphine, noradrenaline (Sato et al., 1973), somatostatin (Williams and North, 1978b) and enkephalin (North and Williams, 1976; Williams and North, 1979a).

### Intracellular Recordings

Studies using intracellular recording techniques have revealed two types of myenteric neurons. This classification was based on the electrical and membrane properties of the neurons (Nishi and North, 1973a; Hirst, Holman and Spence, 1974). One type (Type I or S cell) receives a synaptic input (cholinergic), has a relatively high input resistance, and a low membrane potential. The second type (Type II or

or AH cell) does not receive a fast synaptic input, has a higher resting potential and lower input resistance, and is less excitable than the S cell. An excitatory post synaptic potential is observed in the Type 1 cell as a result of electrical stimulation of the preparation (Nishi and North, 1973a; Hirst, Holman and Spence, 1974) or by distension of the ileum by an intraluminal balloon (Hirst, Holman and Spence, 1974). The e.p.s.p. is abolished by 5-HT and noradrenaline; these substances do not affect depolarizations of a time course similar to that of the e.p.s.p. produced by iontophoresis of acetylcholine. This finding suggests a presynaptic site of drug action for 5-HT and noradrenaline (Nishi and North, 1973b; Henderson and North, 1975). The second type of neuron is characterized by a prolonged hyperpolarizing afterpotential. The afterhyperpolarization persists for 10 - 20 seconds, requires calcium ions and is produced by an increase in potassium conductance (North, 1973; Nishi and North, 1973a).

The main difference between the results obtained with intracellular and extracellular recording techniques in the guinea-pig myenteric plexus was the lack of spontaneous activity recorded with the intracellular electrodes. Wood as early as 1975 explained this apparent discrepancy as a differential selectivity of each method for a particular type of neuron and/or for specific parts of the same neuron. Another explanation was proposed by North and Williams in 1977. They suggested that large extracellular electrodes mechanically deforms the cell membrane causing the membrane potential to reach threshold and discharge action potentials.

#### 1.4.3. Actions of Opiates on the Guinea-Pig Myenteric Plexus

Electrical stimulation of the isolated guinea-pig ileum, or its myenteric plexus longitudinal muscle preparation, induces the release of acetylcholine from intramural nerves and this results in a contraction of the longitudinal muscle (Paton, 1957). Narcotic analgesics inhibit this nerve mediated contraction by inhibiting the release of acetylcholine (Paton, 1957; Schauman, 1957). This action is stereospecific, concentration dependent, and reversed by naloxone in a competitive manner (Kosterlitz and Watt, 1968; Kosterlitz and Waterfield, 1975b). A number of narcotic analgesics have been shown to depress the electrically induced contraction; these include morphine, normorphine, buprenorphine, levorphanol, ketocyclazocine and cyclazocine (discussed below). The myenteric plexus of the rabbit (Greenberg, Kosterlitz and Waterfield, 1970) and cat (Erwin, Nonchoji and Wood, 1978) are not opiate sensitive.

The concentration of a drug which inhibits the nerve mediated contraction of the guinea-pig ileum by 50% is known as the  $IC_{50}$ . The  $IC_{50}$  for morphine, normorphine, levorphanol, ketocyclazocine, cyclazocine and buprenorphine in this preparation was 68, 73, 9.2, 2, 3.6 and 4 nM respectively (Kosterlitz et al., 1972; Kosterlitz et al., 1974; Schulz and Herz, 1976b). Recovery from the depressant effect of high concentrations of normorphine was complete within 90 seconds of washout and a constant depression was observed with repeated exposures at 5 minute intervals (Kosterlitz, Lord and Watt, 1972). The half-time of re-

covery from the inhibition produced by morphine, ketocyclazocine and levorphanol in the guinea-pig ileum was 32, 130 and 268 s respectively (Kosterlitz, Leslie and Waterfield, 1975; Schulz and Herz, 1976b). The half-time of recovery from the inhibition produced by buprenorphine was so very long that it could not be determined with accuracy (Schulz and Herz, 1976b). In general, the more lipophilic the compound the longer its half-time of onset of action and half-time of recovery in the guinea-pig ileum (Kosterlitz, Leslie and Waterfield, 1975). It appears that important differences between the actions of normorphine and morphine in the guinea-pig ileum is the rapid recovery of the twitch after inhibition by normorphine and the reduced tendency of normorphine to develop tachyphylaxis (Kosterlitz, Lord and Watt, 1972).

Naloxone antagonized the inhibitory effect produced by morphine and the other agonists. However, naloxone was much less effective in antagonizing the inhibition of the twitch produced by cyclazocine, ketocyclazocine and buprenorphine than the inhibitions produced by normorphine and morphine (Kosterlitz et al., 1972; Kosterlitz and Watt, 1968; Hutchinson et al., 1975; Schulz and Herz, 1976b).

The action of opiates on the firing rate and membrane properties of single myenteric neurons has been described in section 1.2.1.

#### 1.4.4. Opiate Receptor Studies

Opiate receptors of myenteric neurons appear identical to those receptors subserving analgesia in the central nervous system. This statement is supported by the following evidence. First, the myenteric plexus possesses high affinity opiate binding sites (Gyang and Kosterlitz, 1966; Pert and Snyder, 1973). Second, the potency of an antagonist to inhibit naloxone binding in brain homogenates (Pert and Snyder, 1973) correlates well with its dissociation constant determined pharmacologically in the myenteric plexus (Kosterlitz and Watt, 1968; Kosterlitz, Lord and Watt, 1972). Third, the relative potencies of a series of opiates in depressing the electrically induced contractions of the guinea-pig myenteric plexus longitudinal muscle preparation is comparable to the analgesic potency in man (Kosterlitz and Waterfield, 1975a; Creese and Snyder, 1975). Fourth, the depressant effect of narcotic agonists on the firing rate of myenteric neurons is similar to the action of opiates on central neurons (North, 1979a). Fifth, the inhibitory action of opiates in the myenteric plexus is stereospecific and occurs at low concentrations (Kosterlitz and Waterfield, 1975b; North and Williams, 1977; Dingledine and Goldstein, 1975). Sixth, the action of opiates is reversed with a concentration of naloxone several fold lower than that of the narcotic agonist (Kosterlitz et al., 1972; North and Williams, 1977). Seventh, the antagonist potency of narcotic analgesic drugs determined in morphine-dependent monkeys correlated well with the antagonist potency determined on the guinea-pig ileum (Kosterlitz, Waterfield and Berthoud, 1974). Finally, tolerance

and cross tolerance to the action of narcotic analgesics and the endogenous opioid peptides develops in the guinea-pig ileum (Goldstein and Schulz, 1973; Waterfield, Hughes and Kosterlitz, 1976), as it does in the central nervous system (Pert, 1976).

#### 1.4.5. Opiate Tolerance and Dependence

The first demonstration of tachyphylaxis to opiates in the guinea-pig ileum was made by Paton in 1957. In his experiments he described that the action of morphine to inhibit the electrically induced contraction of the longitudinal muscle was diminished with repeated large concentrations or with lesser concentrations repeated at shorter intervals (less than 15 minutes). This finding was later confirmed by Kosterlitz and Waterfield in 1975 using very high concentrations of morphine (66 - 300  $\mu$ M). They showed that this effect of opiates was related to their anticholinesterase action and was not an opiate receptor mediated event. A second demonstration of tachyphylaxis to morphine in the guinea-pig ileum was made by Shoham and Weinstock in 1974. In their experiments a lower concentration of morphine (50 - 100 nM) was used, and the morphine was allowed to remain in contact with the tissue for 90 minutes. During this time, the inhibitory response to morphine slowly diminished, although the output of ACh remained constant. The authors concluded that the tachyphylaxis observed was due to a supersensitivity of the muscle to ACh much like the denervation supersensitivity observed at other neuromyal junctions (Thesleff, 1960; Fleming, 1976).

Studies on the opiate binding sites during the development of opiate tolerance and dependence revealed no detectable changes in either affinity or number of sites. That is, direct measurement of  $^3$ H-etorphine binding indicated no difference in receptor number or affinity in myenteric plexus strips from morphine dependent guinea-pigs (Cox and Padhya,



1977). However, it is difficult to interpret the results obtained from this type of experiment (see section 1.1.4.).

Two techniques of exposure have been used to induce opiate tolerance and dependence in the guinea-pig ileum. The first technique, in vivo exposure to opiates, was achieved by implanting morphine pellets subcutaneously in guinea-pigs (Schulz and Goldstein, 1973). The second technique, in vitro exposure, was made by incubating segments of guinea-pig ileum with a physiological solution containing morphine for periods of 18 - 22 h (Hammond et al., 1976).

#### In Vivo Exposure

Ileum removed from opiate pretreated animals which is then maintained in an opiate containing Krebs solution (normorphine 200 nM) shows a reduced sensitivity to subsequent applications of morphine. That is, tolerance to the inhibitory effect of morphine on the nerve mediated contraction had developed (Schulz and Herz, 1976a). Cross-tolerance to the inhibitory action of pentazocine, ketocyclazocine (Schulz and Herz, 1976b), and enkephalin (Waterfield et al., 1976) also developed. Exposure to naloxone resulted in a contracture of the longitudinal muscle (Schulz and Herz, 1976a). The contracture was greater when a larger dose of naloxone was used and was the result of a relatively synchronous release of ACh from the myenteric neurons which innervate the longitudinal muscle (Frederickson, Hewes and Aiken, 1976). This contracture has been taken by some investigators to be a manifestation of opiate dependence. A good correlation has been found between the ability of a series of opiates to maintain a state in which naloxone can induce a contracture and their drug dependence liability (Schulz and Herz, 1976b). For exam-

ple, buprenorphine produces a very low level of physical dependence in man (Jasinski et al., 1978) and does not substitute for morphine in maintaining a state giving a naloxone contracture. Likewise, ketocyclazocine and pentazocine only poorly maintain a state in which naloxone produces a contracture (Schulz and Herz, 1976b).

Changes in neuronal sensitivity accompany the development of opiate tolerance and dependence. These experiments were performed with the isolated tissue placed in a Krebs solution free of opiate or with the tissue placed in a solution containing morphine. In the first instance, it was found that the tissues were supersensitive to agents which excite neurons or release ACh, such as 5-HT, potassium chloride, nicotine and  $PGE_1$  (Johnson et al., 1978; Schulz and Herz, 1976a) but subsensitive to agents which inhibit the release of acetylcholine, such as adrenaline, dopamine, noradrenaline and clonidine (Schulz, Cartwright and Goldstein, 1974; Ward and Takemori, 1976; Hughes, Kosterlitz, Robison and Waterfield, 1978). Similar, but less dramatic results have been found with tissue maintained in a morphine containing Krebs solution (Schulz and Herz, 1976a). The mechanism whereby the supersensitivity and subsensitivity to a number of agents was achieved has not been elucidated. It appears that these changes are relatively non-specific and that the continued (chronic) presence of the opiate may lead to a slight depolarization of the cell membrane. This explanation would account for the apparent increase in sensitivity to excitatory agents and a decrease in sensitivity to inhibitory agents. Compatible with such an explanation was the finding by Cox (1978a) that the tension produced by a given electrical stimulus was greater in ileal tissue removed from morphine pretreated guinea-

pigs than in drug naive guinea-pigs.

Extracellular recordings have been made from myenteric neurons removed from morphine pretreated guinea-pigs. The ileum was excised and placed in a physiological solution either with or without morphine. In the latter condition neurons fired at a higher frequency than neurons from drug naive guinea-pigs, and they were also 10 times more sensitive to the excitatory effects of 5-HT (Takayanagi, Sato and Takagi, 1974). Recordings from neurons which were maintained in a morphine containing Krebs solution yielded somewhat different results. No obvious change in the pattern of spontaneous activity was noted but the frequency of the spontaneous activity was less than those of neurons from drug naive animals (North and Zieglgänsberger, 1978). Myenteric neurons treated in this manner responded to the application of naloxone by greatly increasing their spontaneous rate of discharge. The frequency which was often attained was rarely seen in control situations (North and Zieglgänsberger, 1978).

#### In Vitro Exposure

Hammond et al. (1976) found that incubation of ileal segments with morphine (0.35 - 17.5  $\mu$ M) for approximately 20 hours, induced a state of tolerance and dependence. Tolerance was measured as a reduced ability of morphine to inhibit the contractile response and the degree of tolerance was dependent upon the concentration of morphine in the incubation media. Tolerance was induced by levorphanol but not dextrorphan; control incubations were without effect on the sensitivity of the tissue to opiates. Tissue incubated with morphine responded to naloxone with a contracture.

These long term effects of opiates were observed whether incubations were made at 4°C or 37°C, and were similar to those seen in tissues removed from opiate dependent animals (Hammond et al., 1976; Schulz and Herz, 1976a).

Several agents have been shown to modify this development of opiate tolerance and dependence. Concomitant incubation of ileal segments with morphine and cycloheximide (1.9  $\mu$ M) attenuated the degree of tolerance to morphine. Similar findings were obtained when segments of ileum were incubated with morphine and PGE<sub>2</sub>, or morphine and dbcyclic-AMP (Hammond et al., 1976). Agents which were found to enhance the development of opiate tolerance included caffeine and 8-bromocyclic-3', 5'-guanosine monophosphate sodium (Hammond et al., 1976). If the action of caffeine is mediated through an adenylate cyclase-cyclic-AMP mechanism then we would expect dbcyclic-AMP to have an action similar to that of caffeine, because caffeine inhibits the enzyme phosphodiesterase which would result in an increase in the intracellular levels of cyclic-AMP. A further inconsistency includes the results obtained from whole animal studies where it was demonstrated that cyclic-AMP and related agents enhanced the development of opiate tolerance (Ho et al., 1973b).

Similarly, the involvement of protein synthesis in the development of opiate tolerance and dependence is somewhat ambiguous. Hammond (1976) reports that cycloheximide (1.9  $\mu$ M), a protein synthesis inhibitor, reduces tolerance formation in the guinea-pig ileum, yet states that an equieffective level of tolerance was induced at 4°C and 37°C. It is doubtful whether any protein synthesis is being carried out at 4°C (Gutfreund, 1966) and furthermore, other investigators have re-

ported that concentration of cycloheximide necessary to inhibit protein synthesis in this preparation to be 100  $\mu$ M (McKnight, Sosa, Hughes and Kosterlitz, 1978).

#### Time Courses of the Recovery of Opiate Sensitivity After Chronic Exposure

A biphasic recovery from opiate tolerance has been demonstrated in subjects made physically dependent on opiates (see section 1.1.3). A similar biphasic pattern of recovery has been demonstrated in the guinea-pig ileum (Cox, 1978b). Cox has reported that ileum removed from morphine-dependent guinea-pigs and maintained in an opiate containing Krebs solution were highly tolerant to the inhibitory actions of morphine. However, when the opiate was washed from the tissue, tolerance rapidly declined (within 2 hours) until a stable state had been attained. A second protracted phase of tolerance then ensued which persisted 10 hours or more (Cox, 1978b; Johnson, personal communication). Cox referred to the tolerance associated with the first (rapid) and second (protracted) phases of recovery as type 1 and type 2 respectively. Type 1 tolerance has been likened to a state of receptor desensitization and it is in this state that naloxone provokes a withdrawal reaction (a contracture). Within 1 hour of switching to a solution free of normorphine it was found that naloxone had lost its propensity to induce a contracture. Narcotic antagonists do not provoke a withdrawal reaction during type 2 tolerance unless an opiate agonist is previously readministered (Schulz and Herz, 1976a).

The isolated vas deferens from morphine pretreated mice is an example of a tissue which rapidly returns to normal sensitivity when

it is washed with a solution lacking opiate. However, if the yasa are maintained in an opiate containing solution after their removal from the animal, and then tested to a higher dose of normorphine, a 4 fold tolerance is exhibited. In other words, this is an example of a tissue which develops only type 1 tolerance (Cox, 1978b).

### 1.5. SPECIFIC AIMS

The first aim of the present study was to investigate the underlying mechanism of acute opiate action in the guinea pig myenteric plexus. Extracellular recording techniques were used to determine the involvement of the cyclic nucleotides in the depression of neuronal activity by morphine. This part of the study included elevating intracellular cyclic-AMP levels and subsequently testing the preparation for opiate sensitivity.

The second aim of this study was to examine the phenomena of opiate tolerance and dependence at the single neuron level. This part of the study included: (a) an electrophysiological investigation of the effects of long term exposure of myenteric neurons to opiates in vitro or in vivo in order to determine whether tolerance and dependence could be demonstrated; (b) an examination of the effects of drugs which can modify the development of any tolerance and dependence observed; and (c) an investigation of the time course of events recovery from any opiate tolerance and dependence observed.

## 2. METHODS

### 2.1. THE TISSUE

Male guinea-pigs (250 - 500 g) were stunned and bled from the neck. A section of ileum 10 - 15 cm in length, excluding the portion 10 cm oral to the ileo-caecal junction, was rapidly removed and placed in Krebs solution. The luminal content was then gently flushed out. A 2 - 3 cm piece of ileum was cut and gently slipped onto a glass rod (diameter 4 - 6 mm). The diameter of the rod was slightly greater than that of the relaxed ileum. Under the dissecting microscope (10X) the longitudinal muscle with the attached myenteric plexus was carefully stripped away from the circular muscle by gently stroking it with damp cotton wool. The myenteric plexus-longitudinal muscle preparation was gripped by forceps and gently pulled from the remainder of the ileum and placed in a shallow tissue bath.

The method of dissection was originally described by Ambache (1954), and modified later for electrophysiological recordings by Nishi and North (1973a).



## 2.2. IMMOBILIZATION

The longitudinal muscle was pinned with slight stretch and with plexus uppermost. Ganglia were visualized under a dissecting microscope (10 - 60X) and viewed by transmitted light. Electrophysiological recordings from ganglia which contained large swollen cells or those which were concealed by a thick sheet of circular muscle often proved unsuccessful. An area surrounding several suitable ganglia (free of circular muscle and with no swollen cells) was immobilized with several pins (tungsten wire 25  $\mu$ m in diameter and 1 - 2 mm in length). The remaining tissue was cut and discarded.

### 2.3. PERFUSION AND DRUG APPLICATION

Krebs solution of the following composition (mM): NaCl 117, KCl 4.7, CaCl<sub>2</sub> 2.5, MgCl<sub>2</sub> 1.2, NaHCO<sub>3</sub> 25, Glucose 11.5, gassed with 95% O<sub>2</sub> - 5% CO<sub>2</sub>, was continuously pumped over the tissue at a rate of 1 - 3 ml/min. The Krebs solution was warmed in a heating jacket such that the final bath temperature was maintained at 35 - 37°C. Drugs were applied to the tissue by changing the perfusing solution to one which differed only in its content of the drug.

Drugs were prepared fresh daily from stock solutions (1 mM in distilled water) except cyclic-AMP and dbcyclic-AMP which were prepared daily from the solid. Stock solutions of ketocyclazocine and cyclazocine were acidified to pH 5.0 and all stock solutions were kept frozen when not in use. Prostaglandin E<sub>2</sub> was dissolved in ethyl alcohol before addition of the Krebs solution. There was a "dead time" of about 45 - 90 seconds between the time the tap was turned and the arrival of the drug at the bath. In the figures presented in this manuscript, the times indicated are those at which the tap was turned.

The drugs used were: normorphine hydrochloride (Dr. E.L. May), normorphine sulphamate (Dr. E.L. May), normorphine base (Dr. E.L. May), (-)-naloxone hydrochloride (Endo Laboratories), hexamethonium bromide (Sigma), morphine sulphate (Mallinckrodt), dextrorphan tartrate (Roche), levorphanol tartrate (Roche), cycloheximide (Sigma), ketocyclazocine (Dr. R. Schuster), cyclazocine (Dr. R. Schuster), buprenorphine (Reckitt and Colman), PGE<sub>2</sub> (Dr. S. Ehrenpreis), lidocaine (ICN - K&K Labs),

theophylline (1,3-dimethylxanthine) (Sigma), 3-isobutyl-1-methylxanthine (Aldrich), adenosine 3',5'-cyclic monophosphoric acid (Sigma), N<sup>6</sup>, O<sup>2</sup>-dibutyryl adenosine 3',5'-cyclic monophosphoric acid (sodium salt) (Sigma), hyoscine {(-) scopolamine HCl} (Sigma), (+)-naloxone hydrochloride (Dr. A. Jacobson), (+) and (-) 5,9-diethyl-2-(3-furyl methyl)-2'-hydroxy-6,7 benzomorphan hydrochloride (Mr 2266/7) (Boehringer Ingelheim). Morphine antibody was a gift from Dr. B. Wainer.

None of the drugs used significantly altered the pH of the Krebs solution. Concentrations of drugs refer to the final bath concentrations of the compounds listed above.

#### 2.4. THE TISSUE BATH

The tissue bath was similar to the one described by Nishi and North (1973a). A rectangular hole (20 x 40 mm) was cut from the center of a piece of plexiglass (50 x 80 x 1 mm). A coverslip (23 x 50 mm) was then glued over the hole and the trough filled with a thin layer (1 mm) of silicone rubber (Sylgard #184; Dow Corning Corp.).

This clear resin can be transilluminated and is soft enough to allow penetration by fine tungsten pins (for fixing the tissue). A rim of bathtub caulk (5 mm) along the edge of the trough was used to prevent overflowing of solutions and to secure the perfusing tubes and the indifferent electrode.

## 2.5. ELECTROPHYSIOLOGICAL RECORDINGS

A glass suction electrode of tip diameter 40 - 90  $\mu\text{m}$  was used to record extracellular action potentials. The recording electrode was prepared by breaking longer electrodes with a smaller tip diameter. The electrodes were then examined microscopically and those with smooth, flat tips of appropriate size were used. The use of electrodes with a tip diameter greater than 90  $\mu\text{m}$  often resulted in simultaneous recording from more than one neuron, whereas attempts to record with electrodes with tip sizes less than 30  $\mu\text{m}$  were usually unsuccessful. The indifferent electrode was similar but had a tip diameter of 90 - 150  $\mu\text{m}$ ; it was placed near the site of recording. The recording electrode had a resistance of 300 - 1000 K $\Omega$ . It was attached by a piece of rubber tubing to a syringe (5 ml) and suction was applied by withdrawing the plunger. The electrode was gently lowered onto the surface of the ganglia. The suction was sufficient to cause the ganglion to adhere to the electrode tip when the electrode was raised 10 - 20  $\mu\text{m}$ . A third electrode (chlorided silverwire) was placed at a distant site and served to ground the bath.

The recording and indifferent electrodes were filled with Krebs solution; chlorided silver wires led to an AC-coupled preamplifier (Tetronix 122, coupling time constant of 2 ms). The signal was amplified and displayed on an oscilloscope (Tetronix 502A).

Spike activity was gated by amplitude (Mentor Spike Analyzer) and the output was used to determine the number of spikes in a given

time period. This was done either directly from a chart recorder or from a rate analyzer (Fredrick-Haer). Histograms of firing rate were plotted (with respect to time) either by hand from the chart recorder or automatically from the output of the rate analyzer.

## 2.6. 24 HOUR INCUBATIONS - IN VITRO

Pieces of ileum several centimeters in length from newly killed guinea pigs were incubated for approximately 24 hours in either normal Krebs or Krebs solution containing an opiate. Conditions of the incubation were such that the Krebs solution was changed every 4 - 6 hours, gassed with 95% O<sub>2</sub> - 5% CO<sub>2</sub> to maintain a pH of 7.4, and the temperature was kept at approximately 21 - 24°C. In the first of two situations, the ileum was incubated in a normal Krebs solution. Individual myenteric neurons from the incubated preparation were observed with regard to their pattern and frequency of firing, and the acute action of agonists and antagonists on their firing rate. These results were compared with the data obtained from non-incubated preparations. In the second situation ileum was incubated in a Krebs solution which differed only in its content of a narcotic. This Krebs solution was also perfused over the tissue throughout the period of extracellular recording to maintain a constant narcotic environment. Individual myenteric neurons from this incubated preparation were observed with regard to their pattern and frequency of firing, and the effect of agonists and antagonists on their firing rate.

In cases where the ileum was concomitantly incubated with a narcotic and another agent, control incubations were also performed with the other agent alone. A summary list of all incubation solutions used is in Table 3.

Tolerance was tested in two ways. First, the concentration of the narcotic in the incubation solution was sufficient to inhibit

completely the spontaneous activity of non-incubated cells or of cells incubated in normal Krebs solution. Therefore, any neuronal activity recorded after 24 hour exposure to an opiate should reflect tolerance. Second, the ability of even higher concentrations of narcotics to inhibit neuronal firing of cells incubated for 24 hours was tested. The effective concentrations of the narcotics were compared with concentrations which were effective in control incubated tissues.

Signs of dependence were sought in two ways. The firing rate of the myenteric neuron was recorded whilst changing the perfusing solution either to a solution which did not contain morphine, or to a solution which contained both morphine and a narcotic antagonist.



## 2.7. MORPHINE PELLETT IMPLANTED OR INJECTED ANIMALS - IN VIVO

Guinea pigs were injected subcutaneously (cervical area) with morphine sulphate 3 times daily for two days. The following regimen of fixed doses and times was employed: 10 mg/kg, 4 p.m.; 20 mg/kg, midnight; 40 mg/kg, 8 a.m.; 80 mg/kg, 4 p.m.; 100 mg/kg, midnight; 100 mg/kg, 8 a.m. Two hours after the last dose the animal was stunned and exsanguinated.

Guinea pigs were also subcutaneously implanted with 4 morphine base pellets (2 x 75 mg pellets on each flank). The animals were then sacrificed on the third day after implantation. The ileum from both groups of animals was removed as previously described and placed in a Krebs solution which contained morphine (200 - 500 nM). This concentration was chosen because it approximates that in plasma on the third day after pellet implantation (Schulz and Goldstein, 1973).

## 2.8. PLASMA LEVELS OF MORPHINE

Trunk blood was collected from guinea-pigs at the time of death and morphine levels assayed immunologically (courtesy of Dr. Bruce Wainer).

## 2.9. DEFINITIONS

The following terms are commonly referred to in this text.

- inhibition - is defined as a reduction in the spontaneous firing rate of a neuron to a rate which is less than 75% of the control rate.
- excitation - is defined as an increase in the firing of a neuron to a rate which is at least double the control rate.
- slight excitation - an excitation during which the firing rate rises to less than 3 times the original rate.
- marked excitation - an excitation during which the firing rate rises to more than 3 times the original rate.
- slight inhibition - an inhibition during which the firing rate falls to not less than 75% of the original firing rate.
- marked inhibition - an inhibition during which the firing rate falls to a level which is less than 75% of the original firing rate.
- majority - a number greater than  $1/2$  the total number.
- passed off - is an example of a response which does not remain constant at its maximal level.
- rapid - a drug effect was described as rapid in onset if the observed response occurred within 40 s on arrival of the drug to the tissue bath.
- unit - neuron.

## 2.10 ANALYSIS OF DATA

The percentage change in cell firing (Hz) in response to a drug treatment was determined as follows. After a control period of at least five minutes, the tissue was perfused with drug and the change in the rate of discharge recorded. The rate interval analyzer was limited to frequencies less than 10 Hz. Therefore it was necessary, for the purpose of calculating the actual firing in cases of marked excitation, to ascribe a numerical value of 15 Hz to any frequency greater than 10 Hz. The justification for selecting this value was provided by visual observation of the interspike interval meter. A drug effect was not accepted unless a reversal of the effect occurred upon washout of the drug solution.

Results were analyzed by calculation of the mean, standard deviation and standard error of the mean, for the percentage change from control frequencies of firing. The Student's *t* test was used to compare the means of two groups which received different experimental treatments.

### 3. RESULTS

The present results are based on extracellular recordings made from 540 neurons in ganglia removed from 162 animals.

#### 3.1. EXTRACELLULAR RECORDING

Two types of electrical potentials were recorded from the myenteric plexus. They were distinguished from one another on the basis of their duration, amplitude, spike waveform (Fig. 1) and sensitivity to tetrodotoxin and lidocaine. Electrical activity was considered to be of neuronal origin if it lasted 1-3 ms in duration, ranged in amplitude from 50 to 400  $\mu$ V and was reversibly abolished by tetrodotoxin and lidocaine (Sato et al., 1973; Dingledine et al., 1974; 1975; North and Williams, 1977). Neuronal action potentials had a biphasic waveform with a fast component followed by a slower one of opposite polarity (-/+) (Fig. 2). Neuronal action potentials were recorded when the extracellular recording electrode was placed on the surface of a ganglion and suction was applied. It was not possible to record spontaneous neuronal activity without the use of suction; nor were recordings successful with electrodes of tip diameter less than 30  $\mu$ m or when the electrode tip was placed on interconnecting strands between the ganglia or on the muscle itself. Occasionally, when larger tip electrodes (90  $\mu$ m) were used, multi-unit recordings were made possible with one electrode in a single position. Individual neuronal action potentials were distinguished on the basis of their spike waveform and ampli-

tude (Fig. 2). The precise origin of this spontaneous neuronal activity has not been thoroughly investigated; however, it may be attributed to a mechanical deformation of the neuronal membrane caused by the suction electrode (North and Williams, 1977). The second type of electrical activity recorded originated from muscle. The spike waveform was typically biphasic, greater than 20 ms in duration, the amplitude ranged from 400  $\mu$ V to 2 mV; this activity could be recorded only if the electrode were placed on the muscle. These action potentials were resistant to the action of lidocaine and tetrodotoxin (Sato et al., 1973; Dingle-dine et al., 1974). The marked difference in spike duration allowed neuronal action potentials to be easily distinguished from muscle action potentials (Fig. 1).

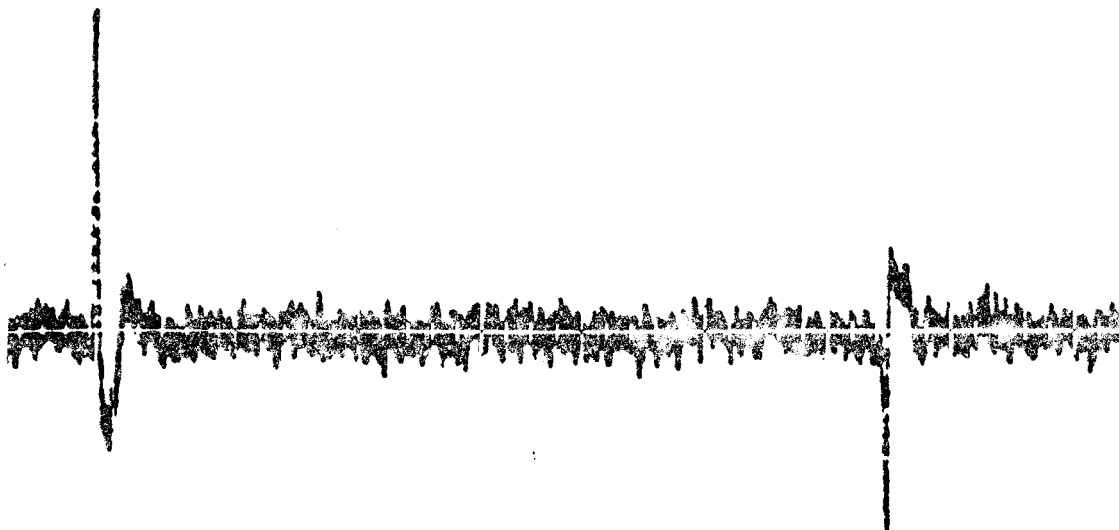
Two types of neurons were distinguished on the basis of their frequency of firing, pattern of discharge, spike waveform and sensitivity to drugs (Sato et al., 1973; 1974; North and Williams, 1977). The most common type encountered fired at a rate of  $3.0 \pm 0.2$  Hz (mean  $\pm$  S.E.M.,  $n=79$ ) and the pattern of discharge was quite variable. In most cases the firing rate declined throughout the period of extracellular recording. Only those units having a stable baseline for at least 15 minutes were included in this study. The spike amplitude ranged from 50  $\mu$ V to 300  $\mu$ V and spike duration was 2-3 ms. The spike waveform was usually biphasic. The second type of neurons (15% of cells) had a higher spike amplitude (150 to 400  $\mu$ V) and fired regularly with low frequency (0.1 - 1 Hz). These findings confirm previously published reports (Dingle-dine et al., 1974; North and Williams, 1977).

Figure 1. Comparison of neuronal and smooth muscle action potentials recorded with an extracellular suction electrode. Figure illustrates one sweep of the trace across the oscilloscope. The first potential change is a smooth muscle action potential; the later two arise from two different neurons. In this and all subsequent oscilloscope photographs, an upward deflection indicates negativity of the recording electrode. Calibration: Horizontal 20 ms. Vertical 200  $\mu$ V. The time constant of the preamplifier was 1 second.





Figure 2. Spike waveforms recorded with a single extracellular electrode in one position. The two distinct waveforms indicate the electrode is simultaneously recording activity from two neurons. Calibration: Horizontal 5 ms. Vertical 100  $\mu$ V. Coupling time constant of the preamplifier was 2 ms. Note the typical biphasic waveforms.



### 3.2. THE ACTION OF OPIATES

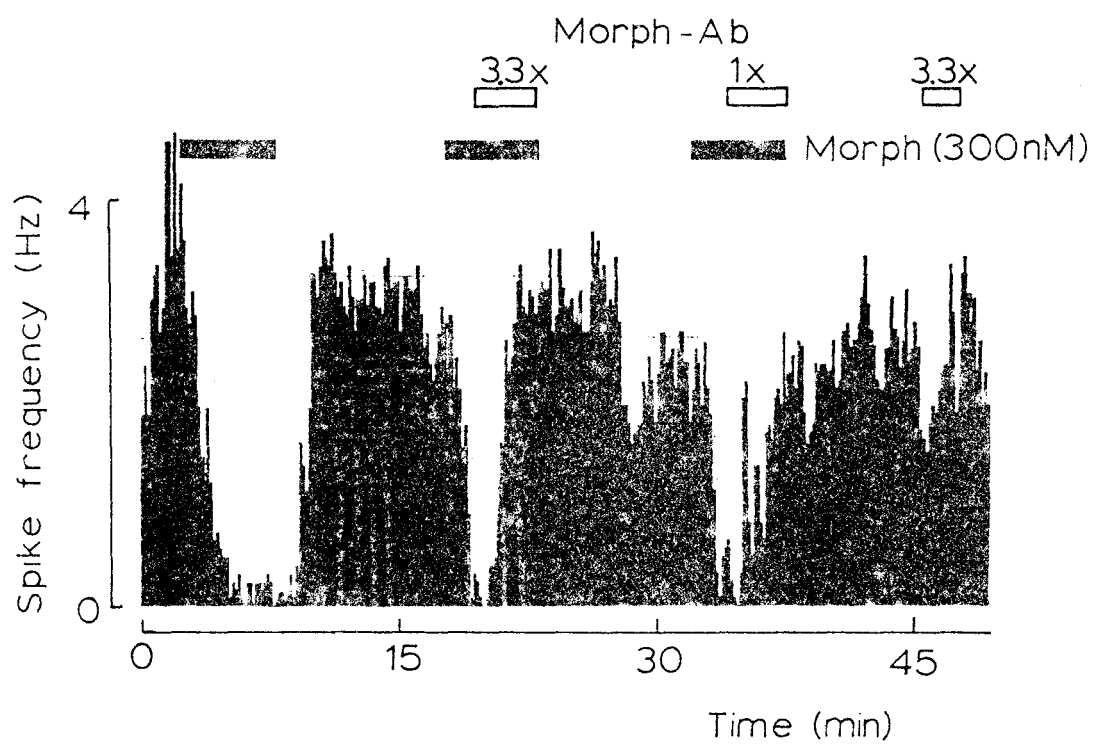
#### 3.2.1. Morphine

Morphine (10 nM - 1  $\mu$ M) inhibited the neuronal firing rate in 86% of the neurons tested (n = 42; 12 animals). Of 11 cells tested with morphine (300 nM), one was unaffected and the degree of inhibition of the remaining cells was  $90\% \pm 3$  (mean  $\pm$  S.E.M.). In a given cell the degree of inhibition was related to the concentration applied. The inhibition reached its maximum within 30 s of the application of the drug, lasted throughout the period of application (2 - 60 min) and was relatively slow to washout. After a 3 min exposure to morphine (300 nM), 6 - 9 min was usually required for the neuronal firing to return to its original rate (Fig. 8a). Tachyphylaxis was not observed with repeated applications of morphine (100 nM - 1  $\mu$ M) for 3 min periods at intervals of 12 min.

#### 3.2.2. Normorphine

Normorphine had an action similar to that of morphine on the neuronal firing rate. Normorphine (1 nM - 1  $\mu$ M) inhibited the neuronal firing rate in 85% of the neurons tested (n = 111; 41 animals). The inhibition was rapid in onset, usually lasted throughout the period of

Figure 3. The inhibition of neuronal firing by morphine and the reversal of inhibition by an antibody to morphine. Ordinate: spike frequency (bin width - 10 s). Abscissa: time. During the periods indicated by the solid and open bars, the solution which perfused the tissue contained morphine (300 nM) and morphine antibody (morph-Ab), respectively. Morphine produced an inhibition in firing which was immediately reversed by the concurrent presence of morph-Ab. If 3.3 times more antibody was added to the bathing solution than was necessary to completely bind all the morphine, then a more rapid reversal was observed. The morph-Ab alone did not cause any effect on the firing rate. The action of morphine on this cell was unusual in that the period of inhibition of cell firing did long exceed the period of application.



application (2 - 60 min) and was readily reversed on washing (Fig. 4). Following exposure (2 min) to normorphine (300 nM), 2-3 min were required for the neuronal firing to return to its control rate. Tachyphylaxis to the inhibitory effect of normorphine on the neuronal firing rate was sometimes observed (section 3.5.1.). Those cells which were inhibited by morphine were also inhibited by normorphine; the compounds appeared to be approximately equipotent. The inhibition of firing caused by normorphine was related to the concentration applied: at 30 nM, 2 cells were unaffected and the mean inhibition of 4 other cells was 58%; at 100 nM, 4 cells were unaffected and the inhibition of 19 other cells was  $80\% \pm 4$  (mean  $\pm$  S.E.M.); at 300 nM, 4 cells were unaffected and the inhibition of 22 other cells was  $82\% \pm 3$ ; at 1  $\mu$ M, 1 cell was unaffected and 6 other cells were inhibited by  $93\% \pm 2$ .

### 3.2.3. Levorphanol

Levorphanol (30 nM - 4  $\mu$ M) inhibited the neuronal firing rate in 84% of the neurons tested ( $n = 18$ ; 5 animals). The inhibition was rapid in onset, persisted throughout the period of exposure (2 - 45 min), and washed out with a time course similar to that of morphine. Neurons which were inhibited by morphine were also inhibited by levorphanol (Fig. 5). Levorphanol (100 nM) inhibited the spontaneous firing of cells by  $91\% \pm 2$  ( $n = 7$ ): this was approximately the same degree of

Figure 4. Concentration dependent inhibition in firing produced by normorphine. During the periods indicated by the solid bars, the perfusing solution was changed to one which contained normorphine (NM). The numbers above the bars indicate the concentrations (nM). Ordinate: spike frequency (bin width - 10 s). Abscissa: time. Normorphine caused an inhibition in firing which was related to the concentration in the bath. During the prolonged exposure to a relatively low concentration of normorphine (50 nM) the inhibitory response 'passed off' with time.

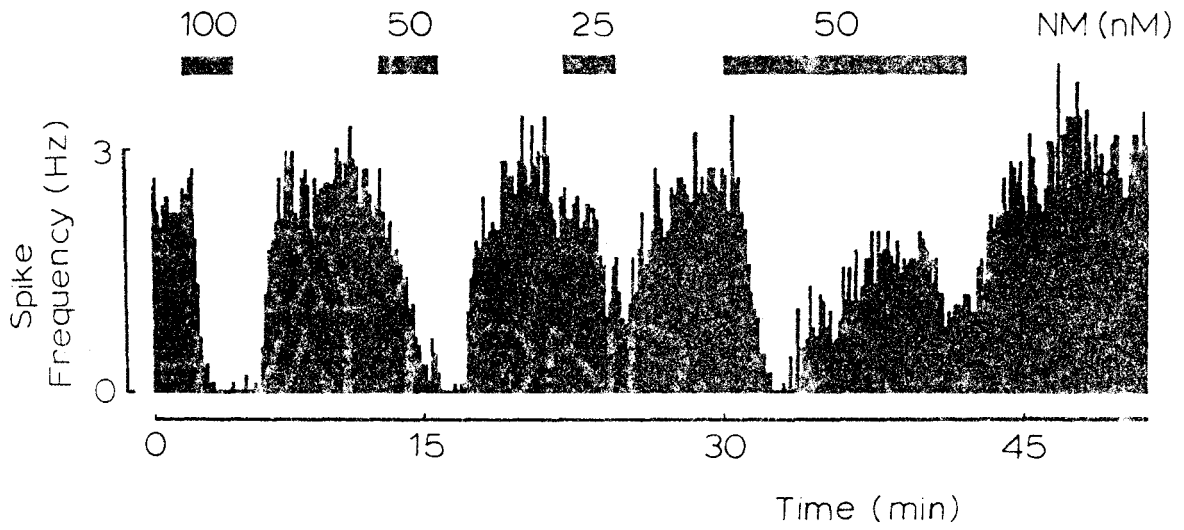
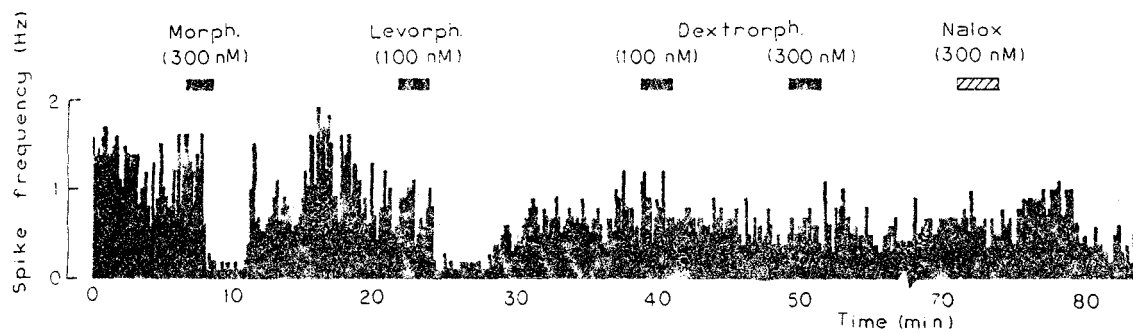




Figure 5. The effect of morphine, levorphanol, dextrorphan and naloxone on a single myenteric neuron. Ordinate: spike frequency (bin width - 10 s). Abscissa: time. The solid bars indicate the periods during which the bathing solution contained either morphine, levorphanol or dextrorphan and the hatched bar indicates the period during which naloxone was added to the perfusing solution. The numbers above the bars indicate the concentration (nM). Morphine and levorphanol caused a marked decrease in the firing rate which was rapid in onset. Dextrorphan and naloxone were without effect.



inhibition as that observed with normorphine (1  $\mu$ M).

#### 3.2.4. Dextrorphan

Dextrorphan (100 nM - 3  $\mu$ M) had no effect on the spontaneous firing rate of 9 cells from 3 animals (Fig. 5; Table 2). These observations confirm those previously reported by Dingledine and Goldstein (1975) and North and Williams (1977).

#### 3.2.5. Cyclazocine

Cyclazocine (3 - 300 nM) inhibited the neuronal firing rate of 53% of the neurons tested ( $n = 19$ ; 6 animals). The inhibition was rapid in onset, persisted throughout the period of application (2 - 8 min) and washed out slowly. The inhibition produced by a 3 min exposure to cyclazocine (100 nM) often outlasted the period of exposure by about 3 - 6 min. Neurons inhibited by cyclazocine were also inhibited by normorphine ( $n = 6$ ) and met-enkephalin ( $n = 2$ ). Three cells were unaffected by cyclazocine (10 nM). Cyclazocine (30 nM) was without effect on 3 cells, excited 1 cell and inhibited 6 cells by  $80\% \pm 5$ .

Table 2. The number of neurons in which firing was inhibited by various narcotic analgesics. Inhibition indicates that the firing rate fell to at least 75% of the control rate.

Drug	Excited	Inhibited	No Effect
Normorphine (1 nM - 1 μM)	2	94	15
Morphine (10 nM - 1 μM)	0	36	6
Levorphanol (30 nM - 1 μM)	0	15	3
Dextrorphan (30 nM - 1 μM)	0	0	9
Ketocyclazocine (30 nM - 1 μM)	1	8	1
Cyclazocine (3 - 300 nM)	2	10	7
Buprenorphine (10 - 100 nM)	5	4	8

A concentration of 100 nM cyclazocine was without effect on 7 cells, and the inhibition of firing of 8 other cells was  $83\% \pm 4$ . In 2 out of 3 cells in which cyclazocine (30 and 100 nM) had no effect on neuronal firing, its presence prevented the inhibition of firing caused by normorphine. That is to say, it acted as a narcotic antagonist.

### 3.2.6. Ketocyclazocine

Ketocyclazocine (30 nM - 1  $\mu$ M) inhibited the neuronal firing rate in 80% of the neurons tested ( $n = 10$ ; 4 animals). The inhibition was rapid in onset but did not readily reverse upon washing (Fig. 6). In a given cell, higher concentrations of ketocyclazocine caused greater degrees of inhibition. Neurons inhibited by ketocyclazocine were also inhibited by normorphine. Ketocyclazocine (100 nM) had no effect of 3 cells, and inhibited the firing of 6 other cells by  $81\% \pm 5$  (mean  $\pm$  S.E.M.).

### 3.2.7. Buprenorphine

The effect of buprenorphine (10 - 100 nM) on the firing rate was variable ( $n = 17$ ; 5 animals). Neurons were excited (30%), inhibited (23%) or not affected (47%) (Table 2). Two cells initially showed a

a biphasic response, excitation followed by inhibition, but with the second application of buprenorphine to the same cell only an excitation was observed (Fig. 7). The excitations recorded were slight, occurred within seconds on entry of the drug to the tissue bath and lasted throughout the period of exposure. No particular tendency was noted for higher concentrations to cause an inhibition or vice-versa. In those cells whose firing was inhibited by buprenorphine, the inhibition was slight, slow in onset (3 - 20 min) and never fully recovered to control levels upon washout of the drug. Prior exposure of the tissue to buprenorphine much reduced the sensitivity of the neurons to the inhibition by normorphine (Fig. 7).

### 3.2.8. Narcotic Antagonists

In only one of 9 cells did naloxone (1  $\mu$ M) increase the firing rate to two times its original value. This confirms previously published findings in which naloxone had no effect on 85% of myenteric neurons tested (n = 30) (Williams and North, 1979a) (see also Fig. 5). Both enantiomers of 5,9-diethyl-2-(3-furylmethyl)-2'-hydroxy-6,7-benzomorphan excited a similar proportion of cells (n = 8). The excitation with any antagonist was always slight (less than three fold increase in rate) and rarely lasted throughout the period of application (3 - 9 min). The lack of stereospecificity of this effects suggests that the excitation was not a consequence of the occupation of the opiate receptor by the antagonist.

Figure 6. A comparison of the effects produced by normorphine and ketocyclazocine on a single myenteric neuron. Ordinate: spike frequency (bin width - 10 s). Abscissa: time. During the periods indicated by the solid and hatched bars, the perfusing solution was switched to one which contained normorphine and ketocyclazocine respectively. Normorphine (300 nM) produced a marked inhibition in the firing rate which rapidly reversed upon washout. The inhibition of firing by ketocyclazocine (180 nM) was also rapid in onset, and slower to washout.



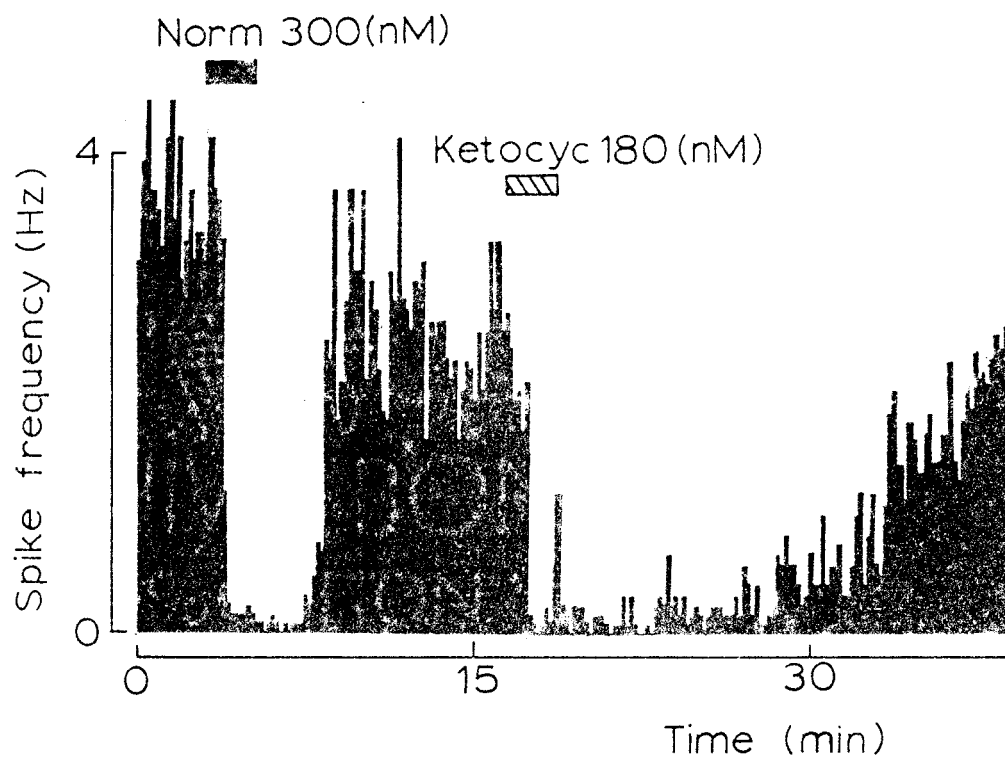
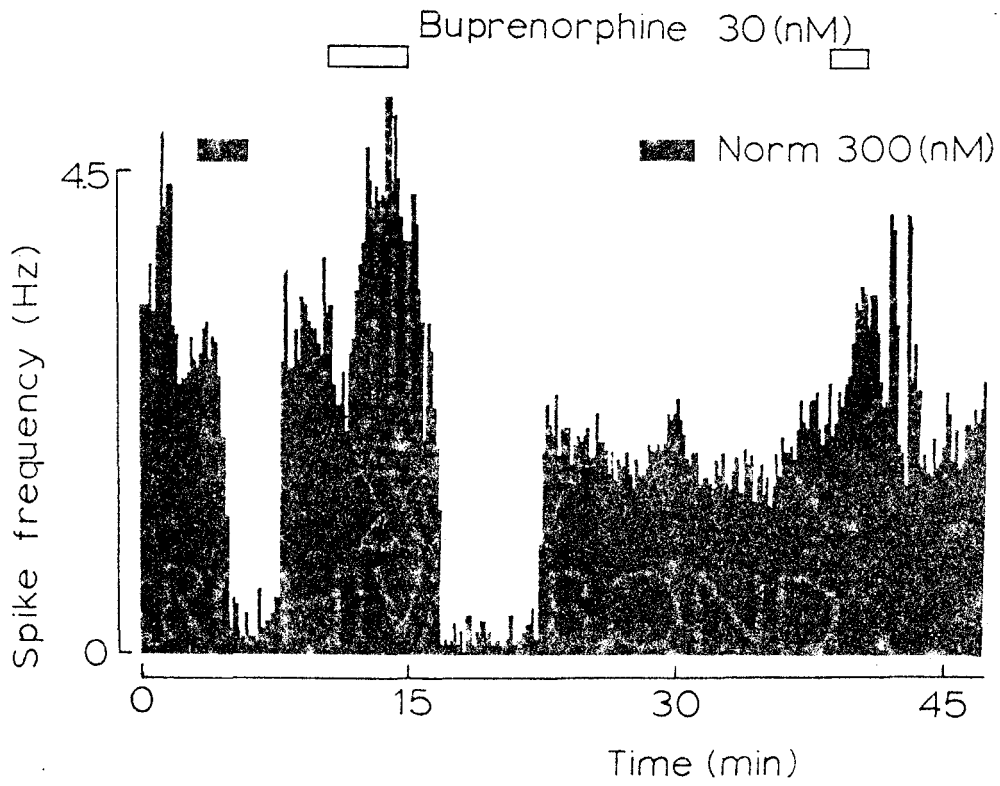


Figure 7. A comparison of the effects produced by normorphine and buprenorphine on a single myenteric neuron. Ordinate: spike frequency (bin width - 10 s). Abscissa: time. During the periods indicated by the solid and open bars, the perfusing solution was switched to one which contained normorphine and buprenorphine respectively. Normorphine (300 nM) caused a marked inhibition of the firing rate. The first application of buprenorphine produced a biphasic response, excitation followed by inhibition; the subsequent application caused only a slight excitation. Exposure of the tissue to buprenorphine prevented the response of the neuron to normorphine.



Naloxone (30 - 300 nM) prevented or reversed the inhibition of firing rate by morphine (100 nM - 1  $\mu$ M) (18 of 18 cells) (Fig. 8a); normorphine (100 nM - 1  $\mu$ M) (9 of 9 cells); levorphanol (100 - 300 nM) (10 of 10 cells) (Fig. 8b); cyclazocine (30 - 100 nM) (2 of 3 cells) and ketocyclazocine (100 nM) (once in one cell). The inhibition of firing by buprenorphine (10 - 100 nM) was not reversed by naloxone (100 nM - 1  $\mu$ M) (3 of 3 cells).

The inhibition of firing produced by morphine was prevented or reversed by an antibody to morphine (Fig. 3) (n = 4). The antibody alone had no effect on the spike firing rate.

Figure 8. Inhibitions of firing produced by morphine and levorphanol and the reversal of these inhibitions by naloxone. Ordinate: spike frequency (bin width - 10 s). Abscissa: time. The solid bars indicate the periods during which the bathing solution contained either morphine or levorphanol in the concentrations indicated, and the hatched bars indicate the period during which naloxone was added (200 - 300 nM) to the perfusing solution. (a) The inhibition of firing produced by morphine (1  $\mu$ M) was marked and long lasting and did not 'pass off' with a prolonged exposure (60 min). The addition of naloxone (200 nM) caused an immediate reversal of the firing rate to control levels. Legend continued on the following page.

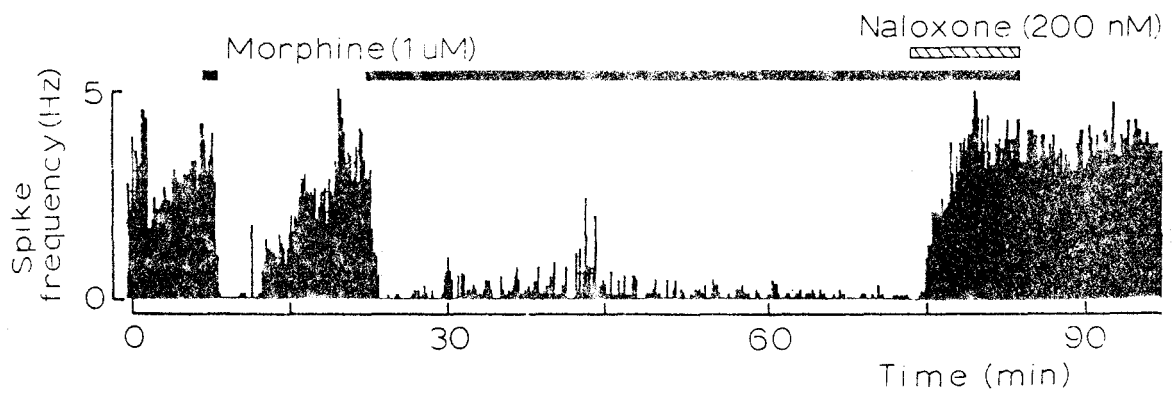
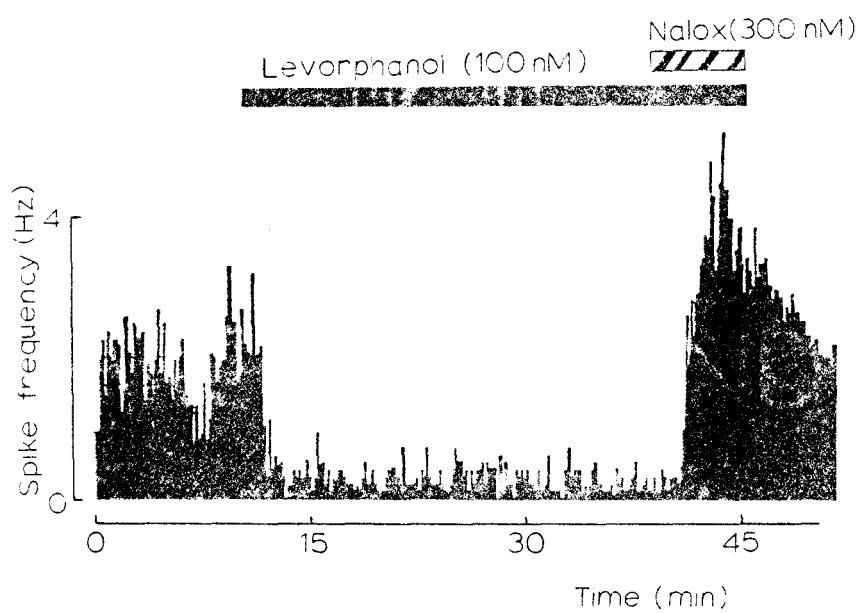


Figure 8b. Levorphanol (100 nM) produced an inhibition in the firing rate and this effect was immediately reversed by the addition of naloxone (300 nM). There was a "rebound" increase in the firing rate during the naloxone reversal which was not observed with morphine reversals.





### 3.3. THE ACTION OF THE CYCLIC NUCLEOTIDES AND RELATED COMPOUNDS ON SINGLE UNIT ACTIVITY

#### 3.3.1. Cyclic-AMP

Cyclic-AMP caused an inhibition of neuronal firing in 80% of the neurons tested; 20% were not affected ( $n = 20$ ). The inhibition was rapid in onset and decline, lasted throughout the period of application (2 - 10 min) and was readily reversed on washout. Increasing the concentration of cyclic-AMP (5  $\mu$ M - 1 mM) caused greater degrees of inhibition. The inhibition of firing was prevented or reversed by the administration of theophylline (50 - 400  $\mu$ M) (Fig. 9). Theophylline alone produced a weak excitation (1.5 - 3x) in the majority of cells tested ( $n = 17$ ). Naloxone (1  $\mu$ M) failed to affect the inhibition produced by cyclic-AMP in the three cells tested.

#### 3.3.2. Dibutyryl Cyclic-AMP

The effects of dbcyclic AMP were more variable than those of cyclic-AMP. Eighteen % of the neurons were excited, 25% of the neurons were inhibited and 57% were unaffected ( $n = 28$ ). No particular tendency was noted for higher concentrations to cause excitation and lower concentrations to cause inhibition, or vice-versa. The inhibition of firing was rapid in onset and decline, lasted throughout the period of

application (2 - 10 min), and readily reversed on washout. The inhibitory effect was concentration dependent (Fig. 10) and when inhibition occurred the effect could be induced repeatedly by repeated applications of the same concentration of dbcyclic-AMP at intervals of 6 minutes. The inhibition of neuronal firing was prevented or reversed by theophylline (50 - 400  $\mu$ M)(n = 3).

### 3.3.3. Prostaglandin E<sub>2</sub>

PGE<sub>2</sub> (10 - 30 nM) increased the firing rate in 80% of the neurons tested (n = 26). It was difficult to perform experiments with concentrations greater than 30 nM because muscle contractions occurred which sometimes dislodged the recording electrode. The increase in firing caused by PGE<sub>2</sub> was rapid in onset; however, it often did not persist throughout the period of exposure (5 - 15 min). The excitations occurred in the presence of hexamethonium (300  $\mu$ M) and hyoscine (1  $\mu$ M) and are, therefore, unlikely to be the consequence of the release of acetylcholine.

Figure 9. The inhibition of firing rate by cyclic adenosine 3',5'-monophosphate (c-AMP) and the reversal of inhibition by theophylline. Ordinate: spike frequency (bin width - 10 s). Abscissa: time. During the periods indicated by the open and solid bars the perfusing solution was changed to one which contained c-AMP and theophylline, respectively. The numbers along side the bars indicate the concentration ( $\mu\text{M}$ ). c-AMP caused an inhibition of firing which was rapid in onset and offset, and which persisted throughout the period of exposure. Theophylline (50 - 300  $\mu\text{M}$ ) rapidly reversed the inhibitory action of c-AMP. The rapidity and degree of reversal was dependent upon the concentration of theophylline.

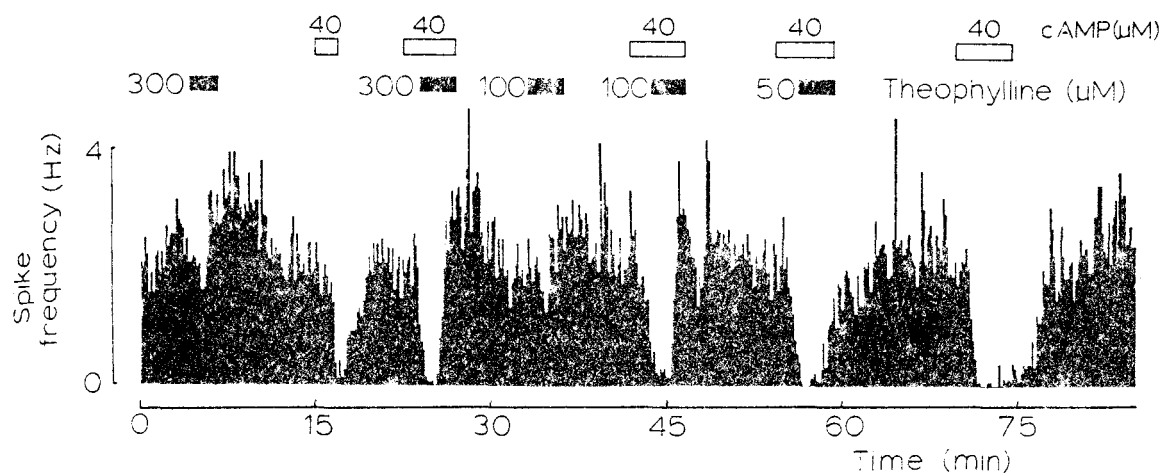
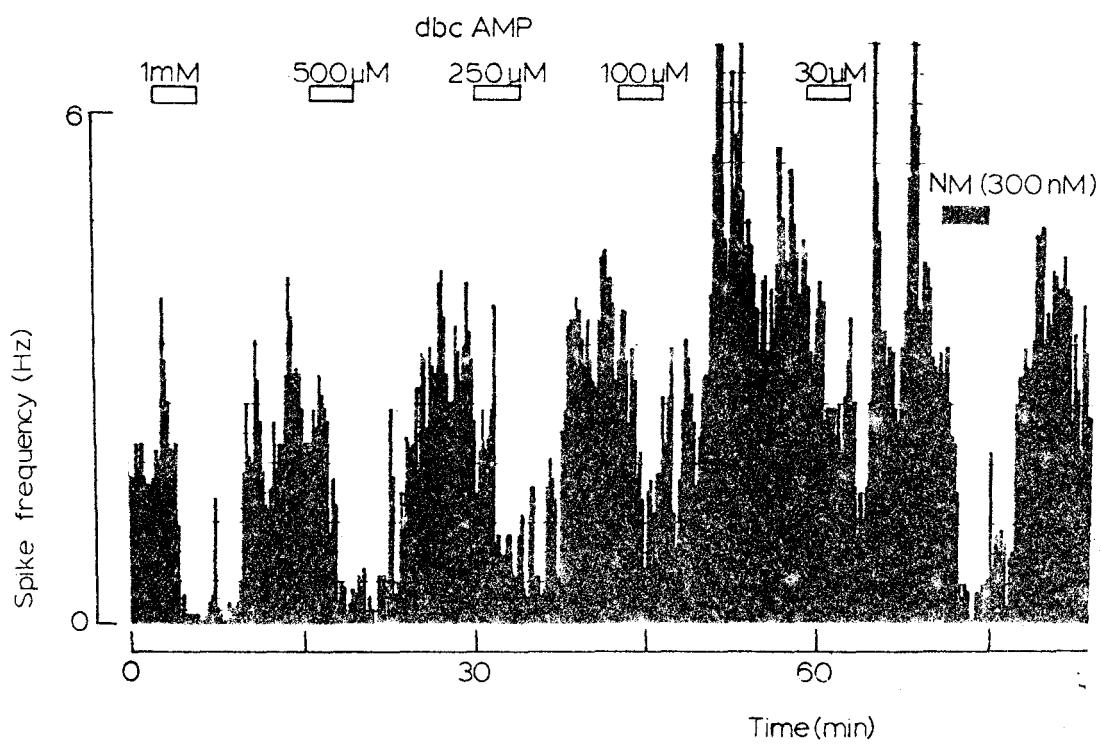


Figure 10. Concentration dependent inhibition of neuronal firing by dibutyryl cyclic adenosine-3',5'-monophosphate (dbcyclic-AMP). Ordinate: spike frequency (bin width - 10 s). Abscissa: time. During the periods indicated by open and solid bars, the perfusing solution contained dbcyclic-AMP (concentrations indicated) and normorphine (NM) (300 nM) respectively. The firing of this neuron was inhibited in a dose dependent manner by dbcyclic-AMP. It was also inhibited by normorphine.



#### 3.3.4. Isobutylmethylxanthine

IBMX ( 6 - 50  $\mu$ M) did not affect the firing rate of 78% of the myenteric neurons; 22% of the neurons tested were excited (n = 28). In these neurons the excitation was slight (2 - 3 x the control firing rate), rapid in onset and did not persist throughout the period of application.

### 3.4. LACK OF EFFECT OF CYCLIC NUCLEOTIDES ON THE INHIBITION OF NEURONAL FIRING BY OPIATES

#### 3.4.1. Cyclic-AMP

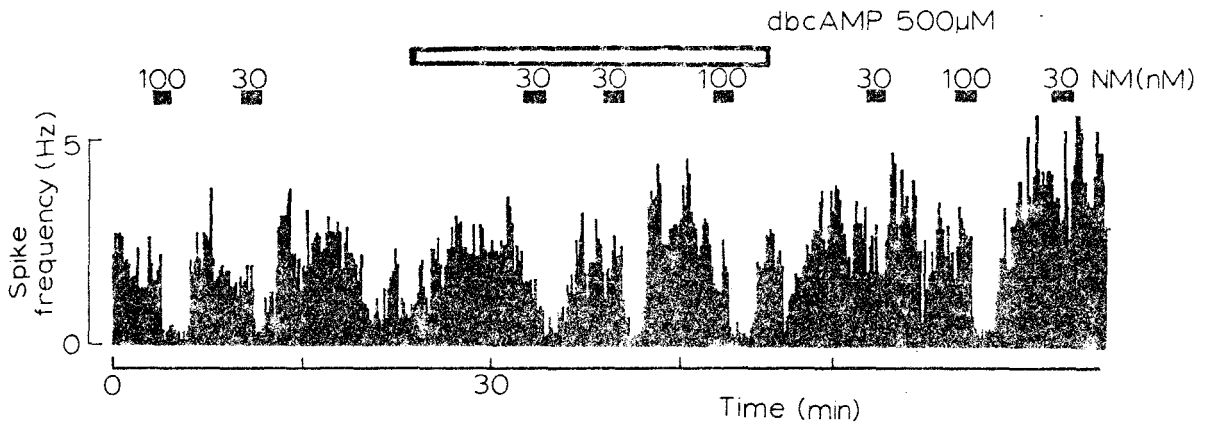
In those neurons in which morphine inhibited firing, the inhibition was not changed when the perfusion solution contained cyclic AMP (5  $\mu$ M - 1 mM) for 6 min prior to and during the presence of the morphine (n = 12). This lack of effect of cyclic-AMP was observed both in cells in which cyclic-AMP itself inhibited firing and in those in which cyclic-AMP had no effect. When the firing was inhibited by morphine, changing to a solution which contained both morphine and cyclic-AMP did not reverse the effects of the morphine (n = 5).

#### 3.4.2. Dibutyryl Cyclic-AMP

Results obtained using dbcyclic-AMP were similar to those reported for cyclic AMP (n = 11) (Figs. 11 and 13).



Figure 11. Lack of effect of dibutyryl cyclic adenosine 3',5'-monophosphate (dbcyclic-AMP) on the inhibition of firing caused by normorphine in a single myenteric neuron. Ordinate: spike frequency (bin width - 10 s). Abscissa: time. During the periods indicated by the solid and open bars, the bathing solution contained dbcyclic-AMP (500  $\mu$ M) and normorphine (concentration indicated in nM), respectively. The neuronal firing rate was depressed by normorphine before, during and after the exposure to dbcyclic-AMP. The sensitivity to normorphine was not reduced during concurrent application of dbcyclic-AMP.



### 3.4.3. Prostaglandin $E_2$

Tissues were exposed to  $PGE_2$  (10 - 30 nM) both prior to (6 - 10 min) and during the application of morphine (300 nM - 1  $\mu$ M). A given concentration of morphine caused the same degree of inhibition of cell firing in the presence and absence of  $PGE_2$  ( $n = 8$ ) (Fig. 12). When cell firing was depressed by normorphine, changing to a solution which contained both normorphine and  $PGE_2$  did not reverse the inhibition ( $n = 5$ ) (Fig. 13).

### 3.4.4. Isobutylmethylxanthine

Tissues were exposed to IBMX (50  $\mu$ M) prior to (6 - 60 min) and during the application of morphine. A given concentration of morphine produced the same degree of inhibition whether in the presence or absence of IBMX ( $n = 12$ ) (Fig. 14a). Likewise, morphine was equieffective in inhibiting neuronal firing even after prolonged exposure (24 h) of the tissue to IBMX (50  $\mu$ M). Also the narcotic antagonist naloxone was without effect on neurons exposed to IBMX (Fig. 14b).

Figure 12. Interaction between prostaglandin  $E_2$  and normorphine on a single myenteric neuron. Ordinate: spike frequency (bin width - 10 s). Abscissa: time. During the periods indicated by the solid and open bars, the bathing solution contained normorphine (NM) and prostaglandin  $E_2$  ( $PGE_2$ ) respectively. Neuronal firing was inhibited by normorphine (300 nM).  $PGE_2$  (30 nM) caused an excitation of the neuron but this did not persist throughout the exposure. Normorphine was equally effective in inhibiting neuronal firing in the presence of  $PGE_2$ .

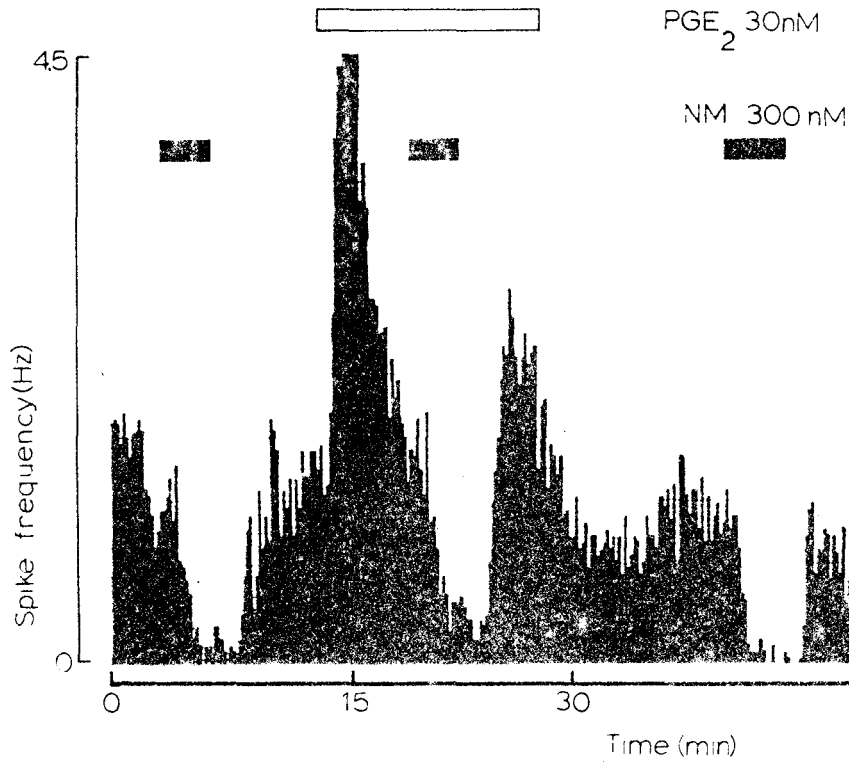


Figure 13. Interaction between normorphine, prostaglandin  $E_2$  and dibutyryl cyclic-AMP. Ordinate: spike frequency (bin width - 10 s). Abscissa: time. During the periods indicated by the solid, open and hatched bars, the perfusing solution was changed to one which contained normorphine (NM), prostaglandin  $E_2$  ( $PGE_2$ ), and dibutyryl cyclic adenosine 3',5'-monophosphate (dbcyclic-AMP) respectively. Normorphine (300 nM) inhibited the firing of this neuron, and the inhibition remained throughout the 30 min period of application.  $PGE_2$  (10 nM) did not excite the cell during the inhibition by normorphine although it did excite the neuron when morphine was not present (not shown); dbcyclic-AMP (400  $\mu$ M) was also without effect.

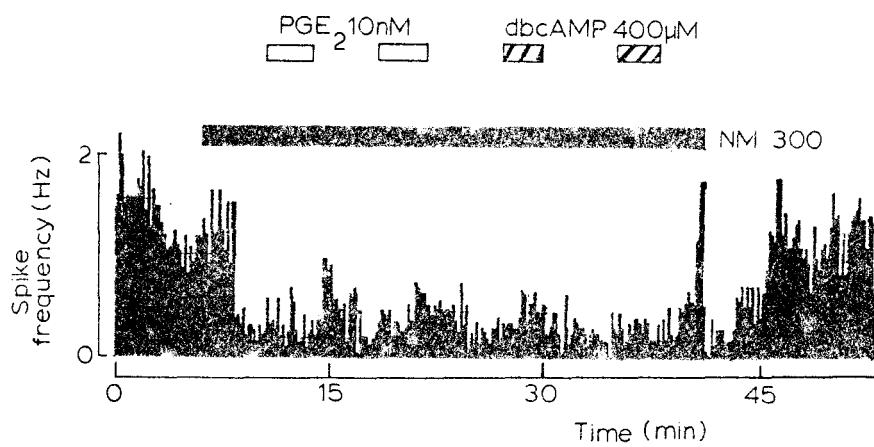


Figure 14. Lack of effect of isobutylmethylxanthine (IBMX) on the inhibition of neuronal firing by morphine. Ordinate: spike frequency (bin width - 10 s). Abscissa: time. (a) The neuronal firing rate was depressed by morphine ( $1\text{ }\mu\text{M}$ ) before, during and after exposure to IBMX ( $50\text{ }\mu\text{M}$ ). The sensitivity to morphine did not appear altered during the concurrent application of IBMX.



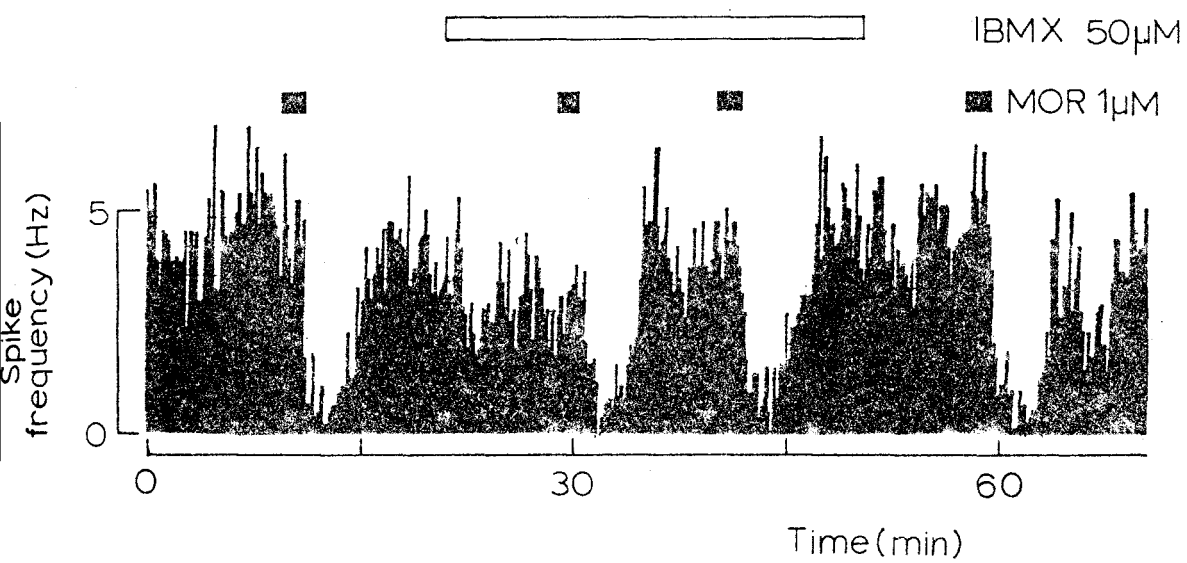
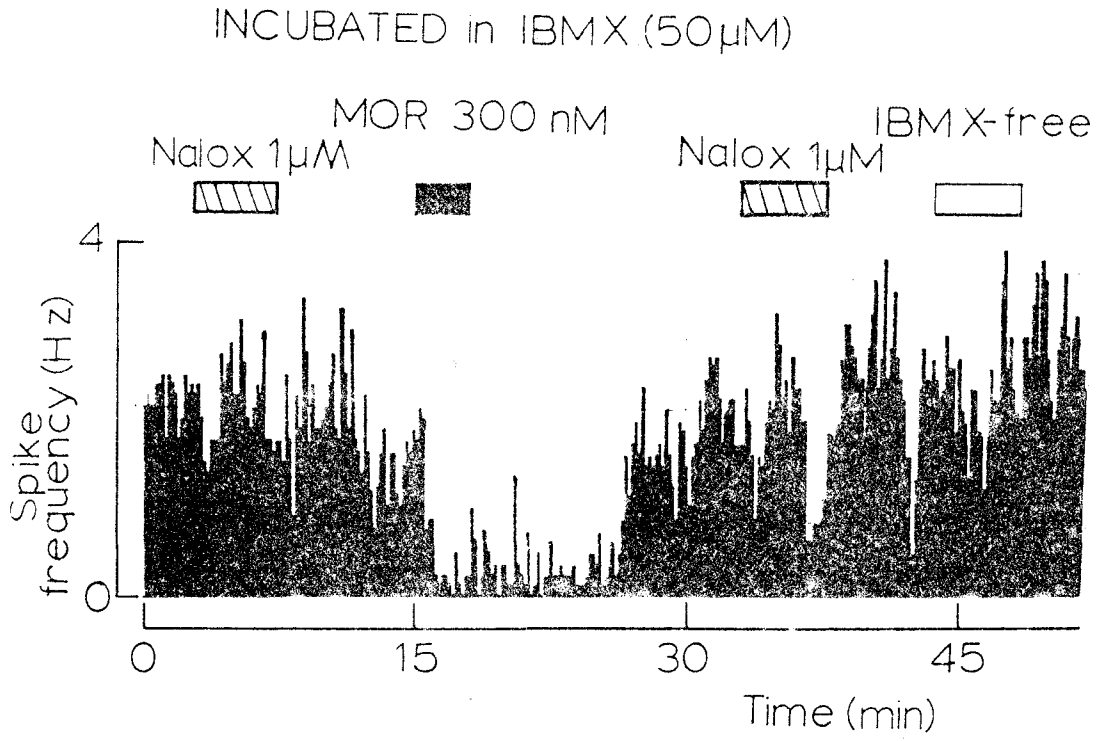


Figure 14b. Prior to this experiment, the isolated ileum had been incubated in a Krebs solution containing IBMX (50  $\mu$ M) for 24 h; the IBMX was present throughout the period of extracellular recording with the exception of the period indicated by the open bar. During the periods indicated by the crosshatched and solid bars, the bathing solution also contained naloxone (1  $\mu$ M) and morphine (300 nM), respectively. Naloxone was without effect, and morphine was as effective as usual in causing an inhibition of firing. Washing out the IBMX did not change the firing rate.



### 3.5. EFFECTS OF PROLONGED EXPOSURE TO OPIATES

#### 3.5.1. Prolonged Exposures (~1 h) whilst Recording from a Single Neuron

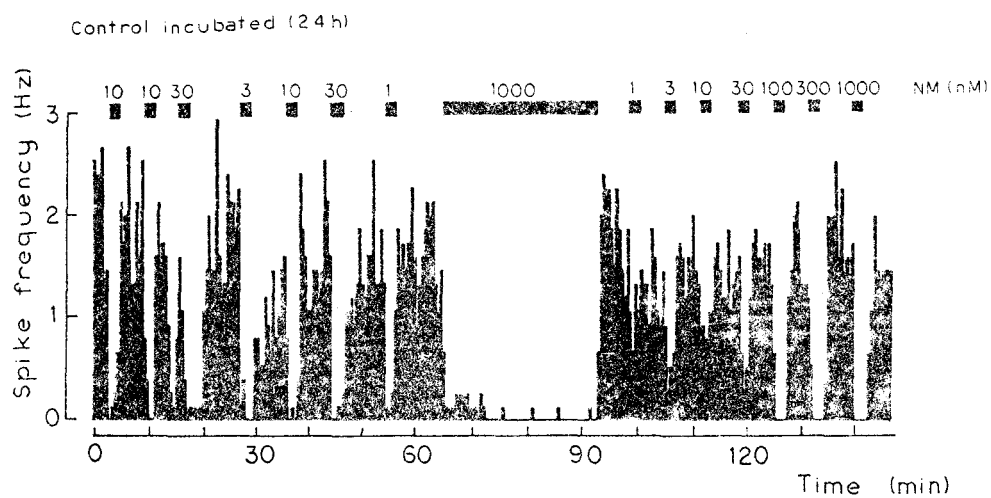
Tachyphylaxis ('acute tolerance') to the inhibitory effect of normorphine on neuronal firing was sometimes observed ( $n = 7$ ). Low concentrations (10 - 50 nM) of normorphine applied for long periods (8 - 60 min) resulted in an inhibition which was not maintained at its maximal level throughout the drug exposure. That is to say, the inhibition of firing 'passed off' during the presence of the normorphine (Fig. 4). The other circumstance in which tachyphylaxis was apparent was following prolonged exposures (30 - 60 min) to a high concentration of normorphine (1  $\mu$ M) ( $n = 6$ ). Following this exposure, the sensitivity of the neuron to previously administered concentrations of normorphine was reduced (Fig. 15).

#### 3.5.2. Prolonged Exposures (~24 h) to Opiates Prior to and During the Recording from a Single Neuron

##### 3.5.2.1. Normal Krebs Solution (control incubations)

Extracellular recordings of myenteric neurons were made from

Figure 15. Inhibition of firing of a myenteric neuron by normorphine. Before the beginning of this recording (time zero), this neuron had been incubated in normal Krebs solution for 24 h. Ordinate: spike frequency (bin width - 30 s). Abscissa: time. During the periods indicated by the solid bars, the solution contained normorphine (NM) in the concentrations indicated (nM). This neuron was one of the most sensitive to the inhibition by morphine which was observed. Following the prolonged exposure (30 min) to a high concentration (1  $\mu$ M), the neuron became somewhat less sensitive to the subsequent effect of the lower concentrations.



tissue which had been incubated 24 h in a normal Krebs solution ( $n = 30$ ; 12 animals). The firing rate was  $2.9 \pm 0.4$  Hz (mean  $\pm$  S.E.M.) and this value did not differ from those of fresh tissue ( $3.0 \pm 0.2$  Hz). The spike waveform and pattern of firing of these neurons did not appear to be different from those tissues which were not incubated. Morphine or normorphine (1 - 300 nM) inhibited neuronal firing in a dose-dependent manner. Normorphine (30 nM) inhibited 12 of 14 cells to which it was applied and the degree of inhibition was  $56\% \pm 8$ ; at 100 nM, 2 cells were unaffected and the inhibition of 10 other cells was  $81\% \pm 6$ ; at 300 nM, one cell was unaffected and the inhibition of 11 other cells was  $89\% \pm 5$ . The time course of the inhibition was the same as that described above (see sections 3.2.1. and 3.2.2.).

Naloxone (100 nM - 1  $\mu$ M) was without effect on 77% of the neurons tested ( $n = 13$ ); 23% were excited (Table 3). The mean excitation by naloxone (200 nM) was 248% ( $n = 3$ ), and did not persist throughout the period of application (3 - 9 min). The excitation was mimicked by both the (+) and (-) isomers of the benzomorphan antagonist (Mr 2267 and Mr 2266 respectively) ( $n = 3$ ).

Microscopic examination of ganglia from tissue which had been incubated for 24 h in Krebs solution (pH 7.4; 22 - 26°C) revealed no gross differences when compared with ganglia from freshly removed tissue.

Table 3. The effect of narcotic agonists and antagonists on the firing rate of myenteric neurons incubated 24 h in a normal Krebs solution or in a Krebs solution containing opiate. An increase in the firing rate (excitation) indicates that the firing rate at least doubled. An inhibition indicates that the firing rate fell to at least 75% of the control rate. The proportion of units inhibited by opiates and excited by narcotic antagonists changed when the tissue was subjected to prolonged exposures to morphine, levorphanol, buprenorphine and ketocyclazocine. For the concentration of opiate used please refer to the text.

Abbreviations:	C <sub>6</sub>	-hexamethonium
	hyos	-hyoscine
	lido	-lidocaine
	cyclohex	-cycloheximide
	Dbc-AMP	-dibutyryl cyclic-AMP
	IBMX	-isobutylmethylxanthine
	levorph	-levorphanol
	dextror	-dextrorphan
	buprenor	-buprenorphine
	ketocyc	-ketocyclazocine



	FRESH TISSUE	INCUBATED TISSUE (24 - 36 h)											
	Control	Control	Morphine					Levorph		Dextror		Buprenor	Ketocyc
			Alone	C6 & Hyos	Lido	Cyclo- hex	Dbc- AMP & IBMX	100 nM	1 μM	100 nM	1 μM		
Percent of neurons inhi- bited by nar- cotics  (1 nM - 1 μM)	86  n=114	85  n=20	0  n=55	0  n=20	0  n=19	0  n=20	0  n=21	14  n=11	-	80  n=15	89  n=9	-	-
Percent of neurons inhi- bited by nar- cotics  (2 - 30 μM)	-	-	22  n=27	10  n=10	23  n=13	11  n=9	18  n=11	-	13  n=8	-	-	8  n=13	55  n=20
Percent of neurons exci- ted by nar- cotic antag- onists  (50 nM - 6 μM)	15*  n=30	23  n=13	79  n=38	81  n=16	85  n=13	60  n=15	50  n=18	86  n=16	100  n=4	15  n=13	40  n=10	6  n=16	65  n=26
Percent of neurons exci- ted by the perfusion of an opiate free Krebs solution	-	-	62  n=21	66  n=3	75  n=4	50  n=2	-	100  n=2	75  n=4	18  n=11	80  n=10	-	100  n=2

\*Williams and North, 1979a

### 3.5.2.2. Morphine

Extracellular recordings from myenteric neurons were made from tissue which had been incubated 24 h in a morphine (1  $\mu$ M) containing Krebs solution (n = 55; animals = 18). The tissue was also continuously exposed to morphine (1  $\mu$ M) throughout the subsequent period of extracellular recording. The firing rate of these neurons was  $1.5 \pm 0.2$  Hz (mean  $\pm$  S.E.M.) and this rate of discharge was significantly different than in those cells which had been incubated without the morphine ( $2.9 \pm 0.4$  Hz) (control incubation) ( $p < 0.05$ , t-test, df = 44). The firing pattern was generally more erratic with distinct bursts of spiking. The spike waveform was not altered. Increasing the morphine concentration to 10 or 30  $\mu$ M was without effect on the firing rate of 21 of 27 neurons; 6 cells were inhibited by  $73\% \pm 6$  (mean  $\pm$  S.E.M.) (Fig. 16). Levorphanol (1 - 10  $\mu$ M) (n = 7) and met-enkephalin (3 & 10  $\mu$ M) (n = 3) had no inhibitory effect on neuronal firing. That is, tolerance and cross-tolerance were apparent.

When the solution was changed to one which differed only in its lack of morphine, the firing rate increased in 13 of 21 neurons. The firing rate increased by  $464\% \pm 65$  and this excitation often did not persist during the entire period of perfusion with a morphine free solution (up to 60 min). When the perfusing solution was changed to one which contained both morphine (1  $\mu$ M) and naloxone (10 nM - 1  $\mu$ M) a marked increase in the firing rate was observed (Fig. 16; Table 3). The excitation by naloxone began immediately (within 40 s) of the arrival of naloxone at the tissue. The naloxone excitation occurred in 30 out of

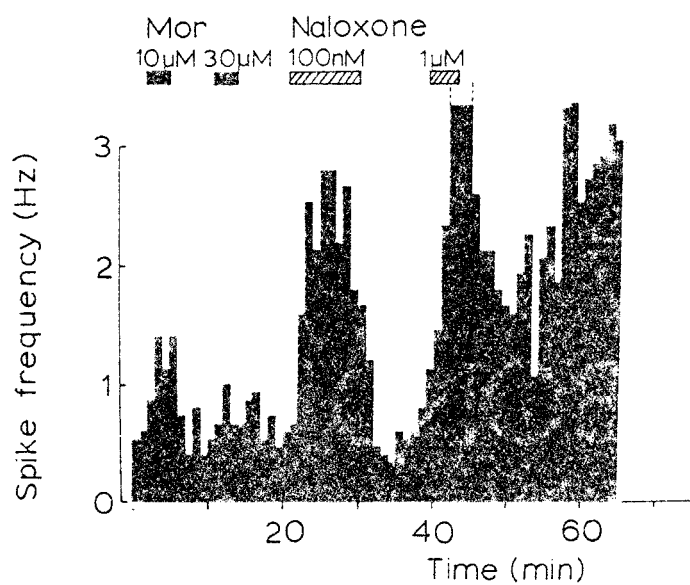
of 38 neurons, its amplitude was dependent on the concentration (10 nM - 1  $\mu$ M), and it sometimes outlasted the period of application (Fig. 16). Naloxone (100 nM) excited 5 cells by an average of  $636\% \pm 191$ ; at 200 nM the average degree of excitation in 8 cells was  $729\% \pm 145$ , and at 1  $\mu$ M the average degree of excitation in 11 cells was  $868\% \pm 150$ . The firing rate of neurons excited by naloxone often reached frequencies which were never observed in control situations (usually more than 10 Hz). The excitation was mimicked by the benzomorphan antagonist Mr 2266 (100 nM - 1  $\mu$ M) but not its (+) -enantiomer Mr 2267 (100 nM - 1  $\mu$ M). (+) -naloxone did not affect the firing rate ( $n = 3$ ). The excitation was reproducible in 73% of the cells when short exposures (3 min) to naloxone were used. However, 27% of the neurons tested showed progressively declining excitations upon the second and third applications. When naloxone was applied for a prolonged period of time (120 min) the excitation 'passed off' during the presence of naloxone.

#### 3.5.2.3. Levorphanol

Extracellular recordings were made from myenteric neurons in tissues which had been incubated 24 h in a Krebs solution containing levorphanol (100 nM or 1  $\mu$ M). With the lower concentration, results are based on recordings made from 20 units taken from 9 animals. The neurons were continuously exposed to levorphanol (100 nM) throughout the period of incubation and the subsequent period of extracellular re-

Figure 16. The effect on a myenteric neuron of prolonged exposure to morphine. Before beginning the recording (time zero), this neuron had been exposed continuously to a solution containing morphine ( $1\text{ }\mu\text{M}$ ) and this solution continued to perfuse the tissue throughout the period of recording. During the periods indicated by the solid and hatched bars, the perfusing solution was switched to one which contained morphine and naloxone respectively. Ordinate: spike frequency (bin width - 60 s). Abscissa: time. Tolerance to the inhibitory effect of morphine had developed--that is, increasing the morphine concentration to  $30\text{ }\mu\text{M}$  was without effect on the firing rate. Naloxone ( $100\text{ nM}$ ) caused an increase in spike frequency. When a high concentration of naloxone ( $1\text{ }\mu\text{M}$ ) was used the frequency of firing exceeded  $10\text{ Hz}$ , (period within the dotted lines) and this excitation persisted beyond the period of exposure. As a consequence of the naloxone ( $1\text{ }\mu\text{M}$ ) application the longitudinal muscle was observed to undergo strong contractions which led to the dislodgement of the recording electrode from the ganglion at time 64 min.

Incubated in Morphine (1 $\mu$ M)



coding. The mean firing rate of these neurons was  $1.3 \pm 0.2$  Hz ( $n = 18$ ) and this rate was significantly different from that observed in control incubations ( $2.9 \pm 0.4$  Hz) (mean  $\pm$  S.E.M.;  $p < 0.05$ , t-test,  $df = 39$ ) and the firing pattern was generally more erratic with distinct bursts of firing. Nine out of eleven units tested were not affected by increasing the levorphanol concentration to 300 nM or 1  $\mu$ M (Fig. 17). When the levorphanol was removed from the perfusing solution during the period of extracellular recording, the firing rate of 2 of 2 neurons increased (Table 3). Similarly, an excitation was observed in 14 of 16 neurons when the perfusing solution was changed to one which contained either naloxone (100 nM - 1  $\mu$ M) or Mr 2266 (100 nM - 1  $\mu$ M) in addition to levorphanol (100 nM). Naloxone (200 nM) excited 7 of 10 neurons by  $794\% \pm 167$ . The excitation occurred almost immediately when the drug reached the tissue, persisted throughout the period of application (3 - 10 min), and rapidly declined when the solution was changed back to one containing only levorphanol (100 nM). The excitation was repeatable with subsequent applications of naloxone. Mr 2267 (100 nM - 1  $\mu$ M) and (+) -naloxone were without effect on the firing rate.

Tissues were also incubated in solutions containing levorphanol (1  $\mu$ M) (4 animals). The mean firing rate ( $1.7 \pm 0.3$  Hz) ( $n = 11$ ) and pattern of firing were similar to those of neurons incubated in levorphanol 100 nM. Seven of 8 neurons tested were not inhibited by increasing the levorphanol concentration to 2 - 10  $\mu$ M. Normorphine (1 - 10  $\mu$ M) was without effect on the firing rate ( $n = 5$ ); that is to say, cross-tolerance had developed. When the levorphanol was removed from the perfusing solution during the period of extracellular recording, the firing

rate of 3 of 4 neurons increased by 775% (Table 3). When naloxone or Mr 2266 was added to the perfusing solution an excitation was observed similar to that described above for neurons incubated in levorphanol (100 nM) (Table 3).

#### 3.5.2.4. Dextrorphan

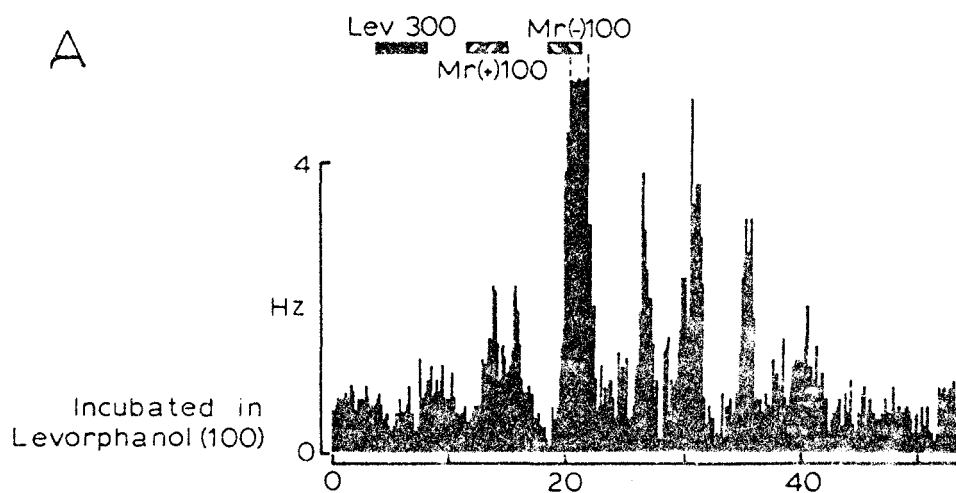
These results are based on extracellular recordings from myenteric neurons in tissue which had been incubated in dextrorphan (100 nM or 1  $\mu$ M). The firing rate ( $2.3 \pm 0.4$  Hz) ( $n = 24$ ), spike waveform and firing pattern of these neurons did not differ markedly from those of control incubated tissue ( $p > 0.05$ , t-test,  $df = 45$ ). Neurons treated in this manner were still sensitive to the inhibitory action of normorphine (30 - 300 nM). Normorphine (300 nM) inhibited 12 of 15 cells incubated in dextrorphan (100 nM), and the degree of inhibition was  $83\% \pm 3$ . Normorphine (300 nM) inhibited 8 of 9 cells incubated in dextrorphan (1  $\mu$ M) by  $84\% \pm 5$ . The inhibition was rapid in onset and offset, persisted throughout the period of application, and these findings were not distinguishable from the effect of normorphine on control incubated tissue.

In the case of dextrorphan (100 nM), changing to a dextrorphan free solution resulted in an excitation in 2 out of 11 cells. The excitation was slight (approximately doubling of firing rate). The effect was observed of changing to a solution which contained dextrorphan (100 nM) and one or other of the isomers of benzomorphan. The (-) isomer, (Mr 2266) (100 nM - 1  $\mu$ M) caused a small excitation in 2 of 13 cells. The (+) isomer (Mr 2267) (100 nM - 1  $\mu$ M) had no effect on the firing rate of 4 cells (Fig. 17). (+) Naloxone (100 nM - 1  $\mu$ M) also had no effect on firing when added to the dextrorphan containing solution. However, tissues incubated with the higher concentration of dextrorphan (1  $\mu$ M) responded differently. Removing the dextrorphan from the per-

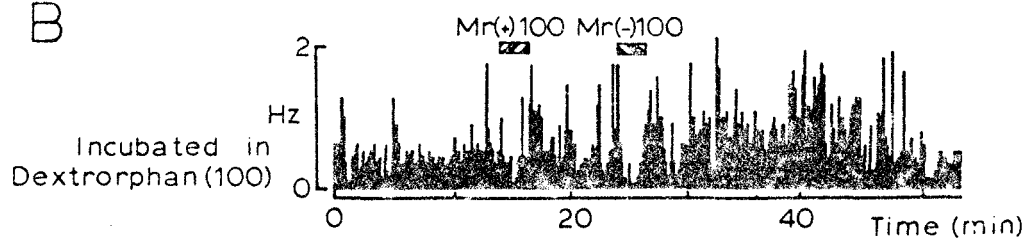


Figure 17. The effect of the narcotic antagonist Mr 2266 on myenteric neurons exposed to levorphanol or dextrorphan for 24 h. Before beginning the recording (time zero), this neuron had been exposed continuously either to a solution containing levorphanol (100 nM) or to one containing dextrorphan (100 nM) for 24 h; these solutions continued to perfuse the tissue throughout the period of recording. During the periods indicated by the solid and hatched bars, the perfusing solution was changed to one which contained levorphanol and either Mr 2266 or Mr 2267. The numbers above the bars indicate the concentration (nM). Ordinate: spike frequency (bin width - 10 s). Abscissa: time. (a) A neuron which had been incubated in levorphanol (100 nM). The firing rate was not inhibited by the application of a high concentration of levorphanol (300 nM). The inactive isomer of the benzomorphan antagonist Mr 2267 (100 nM), (Mr (+)), was without effect on the firing rate whereas Mr 2266 (100 nM), (Mr (-)), caused an excitation. Even after the washout of the antagonist the firing rate rose and fell for several min. (b) Mr (+) and Mr (-) were without effect on neurons previously incubated in dextrorphan (100 nM) for 24 h.

A



B



fusing solution resulted in an increase in the firing rate in 8 of 10 neurons. Naloxone (100 nM - 1  $\mu$ M) and Mr 2266 (100 nM - 1  $\mu$ M) excited 4 of 10 neurons (Table 3) which were incubated with dextrorphan (1  $\mu$ M). The mean excitation produced by naloxone (200 nM) was 545% ( $n = 4$ ), and this value was not significantly different from the value ( $692\% \pm 107$ ) obtained with levorphanol (1  $\mu$ M) incubations ( $p > 0.05$ , t-test,  $df = 11$ ). The excitation was rapid in onset and persisted throughout the period of application (3 - 6 min). An excitation was not observed with either (+)-naloxone (100 nM - 1  $\mu$ M) or Mr 2267 (100 nM - 1  $\mu$ M) ( $n = 5$ ). These results suggest that the higher concentration of dextrorphan was producing changes analogous to those caused by morphine or levorphanol.

### 3.5.2.5. Buprenorphine

The results are based on extracellular recordings from 22 neurons (6 animals) from tissue which had been incubated for 24 h in Krebs solution containing buprenorphine (30 nM). The neurons were continuously exposed to buprenorphine (30 nM) throughout the subsequent period of extracellular recording. The firing rate of these neurons ( $n = 12$ ) was  $2.9 \pm 0.5$  Hz, and this value did not differ from that observed in tissue which had been incubated without buprenorphine ( $p > 0.05$ , t-test,  $df = 33$ ). Increasing the concentration of buprenorphine to 100 nM was without effect on the firing rate. Morphine (2 - 30  $\mu$ M) had no effect on firing of 12 of 13 neurons (Fig. 18; Table 3). It is

possible that this represents cross-tolerance to morphine. However, in the naive tissue (see section 3.2.7.), buprenorphine inhibited the firing of only about one-quarter of cells, yet acted as a morphine antagonist in every case ( $n = 5$ ). The blockade of morphine inhibition after prolonged presence of buprenorphine may, therefore, be an expression of buprenorphine's antagonist properties - to which tachyphylaxis has not developed.

When the perfusing solution was changed to one which contained both buprenorphine (30 nM) and naloxone (100 nM - 1  $\mu$ M) or buprenorphine (30 nM) and Mr 2266 (100 nM - 1  $\mu$ M), the firing rate was not affected in 15 of 16 cells (Fig. 18); one neuron was excited (Table 3).

#### 3.5.2.6. Ketocyclazocine

The results are based on recordings made from 39 neurons from 9 animals. Tissue was kept in Krebs solution containing ketocyclazocine (300 nM & 1  $\mu$ M) for 24 h and perfused with the same solution during the period of extracellular recording. The firing rate was  $1.9 \pm 0.2$  Hz (mean  $\pm$  S.E.M.) ( $n = 26$ ) and this value was not different than the value ( $2.9 \pm 0.4$  Hz) obtained from tissue incubated in a similar solution except that it lacked ketocyclazocine ( $p > 0.05$ , t-test,  $df = 47$ ). Increasing the concentration of ketocyclazocine to 5  $\mu$ M was without effect on the firing rate (Fig. 19), whereas in control incubations this concentration inhib-

ited spike discharge. Morphine ( $2\text{ }\mu\text{M}$ ) had no effect on the firing rate or decreased it by less than 50% ( $n = 8$ ). Increasing the concentration of morphine (or normorphine) up to  $6\text{ }\mu\text{M}$  resulted in an inhibition ( $77\% \pm 5$ ) of neuronal firing in 11 of 20 neurons (Fig. 19): this contrasts with the effect of normorphine on tissue incubated with levorphanol or morphine, in which neuronal firing was rarely inhibited by normorphine ( $5\text{ }\mu\text{M}$ ). Neuronal excitations were observed when the perfusing solution was changed to one which contained naloxone ( $n = 16$ ), cyclazocine ( $n = 2$ ), or Mr 2266 ( $n = 8$ ), in addition to ketocyclazocine (Table 3). The excitation by naloxone ( $200\text{ nM} - 2\text{ }\mu\text{M}$ ), or Mr 2266 ( $200\text{ nM} - 2\text{ }\mu\text{M}$ ) was marked (3 - 10 fold) (Fig. 19) and similar to that caused by naloxone in morphine-incubated tissue. It began immediately upon arrival of the antagonist at the tissue, was concentration dependent and often repeatable on the same cell. The excitation produced by naloxone ( $200\text{ nM}$ ) was  $484\% \pm 166$  ( $n = 5$ ). Larger concentrations of naloxone ( $1$  and  $2\text{ }\mu\text{M}$ ) gave excitations of  $636\% \pm 162$  ( $n = 7$ ) and  $742\% \pm 194$  ( $n = 5$ ), respectively; these excitations were comparable to those caused by naloxone in tissues incubated with levorphanol ( $100\text{ }\mu\text{M}$ ) ( $794\% \pm 167$ ) and morphine ( $1\text{ }\mu\text{M}$ ) ( $729\% \pm 145$ ) (mean  $\pm$  S.E.M.).

Figure 18. The effect on a myenteric neuron of prolonged exposure to buprenorphine. Before beginning the recording (time zero) this neuron had been exposed continuously to a solution containing buprenorphine (30 nM) for 24 h and this solution continued to perfuse the tissue throughout the period of recording. During the periods indicated by the open, solid and hatched bars, the perfusing solution was changed to one which contained cyclic-AMP (250  $\mu$ M), normorphine (3 & 30  $\mu$ M) and naloxone (200 nM), respectively. Ordinate: spike frequency (bin width - 10 s). Abscissa: time. Cyclic-AMP (250  $\mu$ M) caused an inhibition in firing rate which was maintained throughout the period during which the cyclic-AMP was in the bath. Application of normorphine (3 & 30  $\mu$ M) or naloxone (200 nM) did not significantly affect the firing rate.

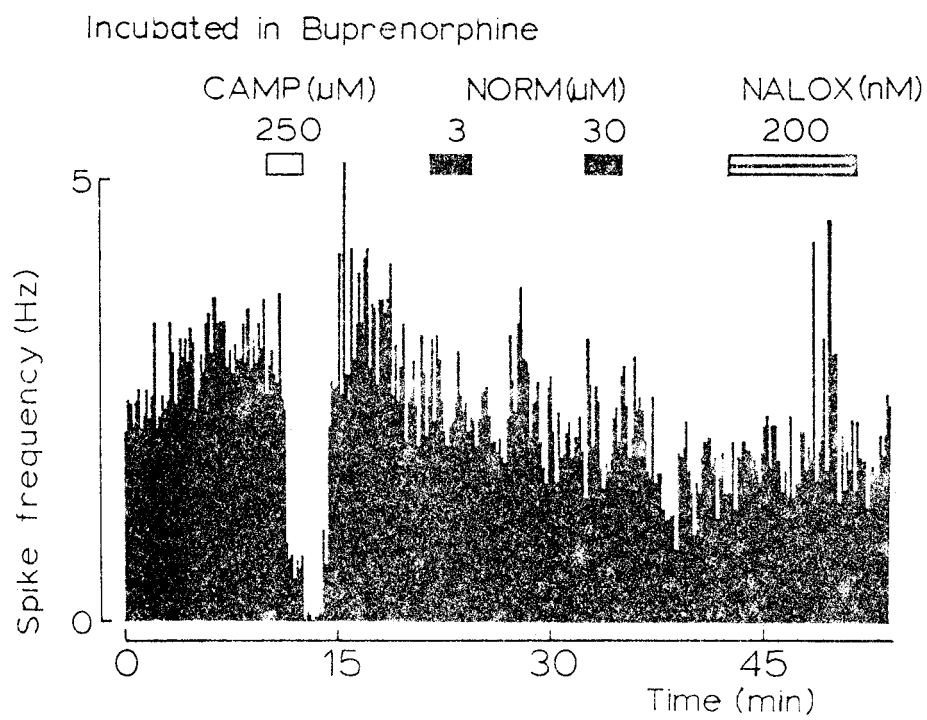


Figure 19. The effect on a myenteric neuron of prolonged exposure to ketocyclazocine. The tissue had been exposed to ketocyclazocine ( $1\text{ }\mu\text{M}$ ) for 24 h and was continuously bathed with the same solution throughout the subsequent period of recording except for the time indicated by the open bar. The solid, diagonally hatched, vertically hatched, and horizontally hatched bars indicate the periods during which the perfusing solution was changed to normorphine ( $3 - 6\text{ }\mu\text{M}$ ), ketocyclazocine ( $5\text{ }\mu\text{M}$ ), naloxone ( $200\text{ nM}$  &  $1\text{ }\mu\text{M}$ ) and cyclazocine ( $1$  &  $3\text{ }\mu\text{M}$ ), respectively. Ordinate: spike frequency (bin width - 10 s). Abscissa: time. (a) Normorphine ( $3$  &  $6\text{ }\mu\text{M}$ ) produced an inhibition in firing rate which was dose-dependent. However, such concentrations of normorphine would completely inhibit cell firing in control incubated cells (see Fig. 15); that is to say, some cross-tolerance to normorphine had developed. Ketocyclazocine ( $5\text{ }\mu\text{M}$ ) was without effect. Naloxone ( $200\text{ nM} - 1\text{ }\mu\text{M}$ ) produced a concentration dependent excitation.



Incubated in Ketocyclazocine

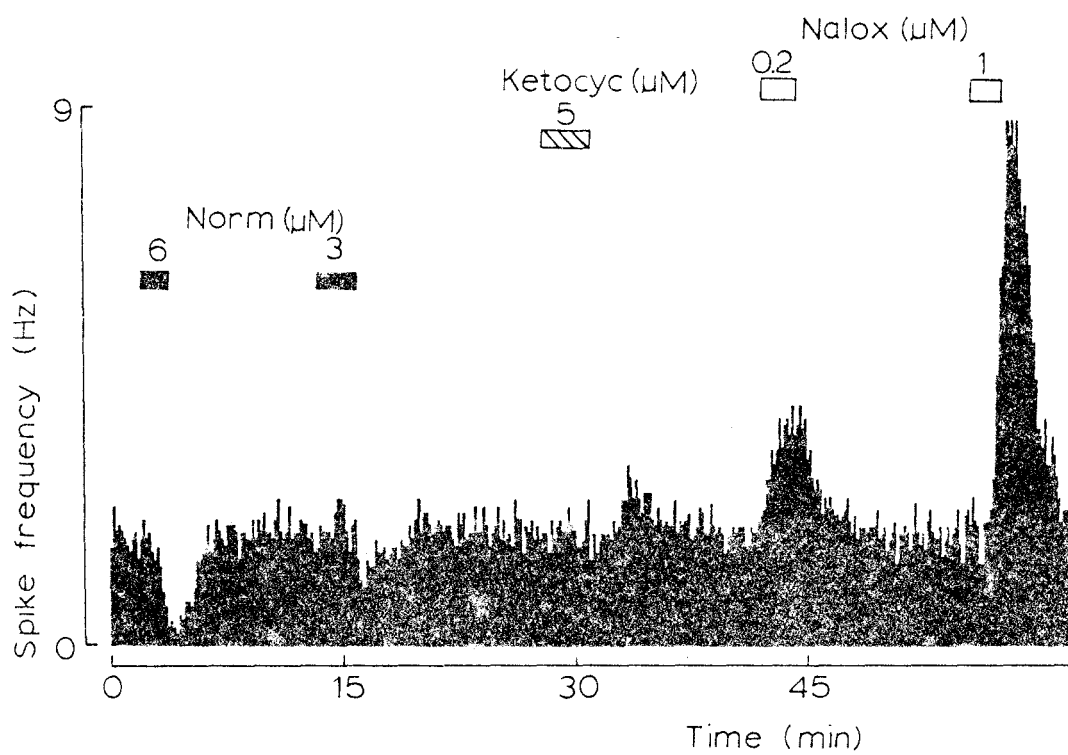
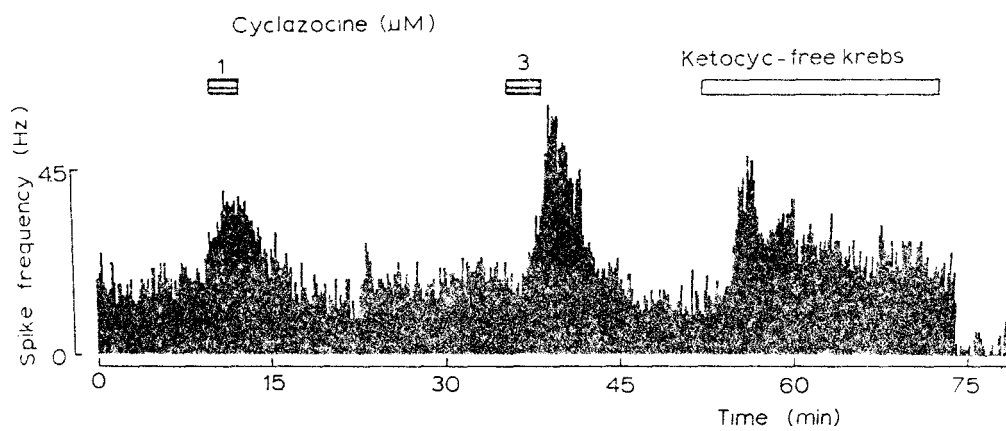


Figure 19b. Same neuron as in (a). Perfusing the tissue with cyclazocine (1 - 3  $\mu$ M) or a ketocyclazocine free Krebs solution also produced an excitation, however, the excitations produced were not as marked as those caused by naloxone.

Incubated & perfused in Ketocyclazocine



### 3.5.3. Prolonged Exposures In Vivo (3 days)

Samples of blood were collected from guinea-pigs subcutaneously injected ( $n = 11$ ) with morphine sulfate or implanted ( $n = 8$ ) with morphine pellets (see Methods). The average serum concentration of morphine from pellet implanted animals ( $n = 4$ ) was  $4.7 \mu\text{M}$  while that of the injected animals ( $n = 6$ ) was  $12.7 \mu\text{M}$ . Samples of blood were collected 2 h after the last dose was given to the injected group and on the third day following implantation. The average weight loss for the injected and implanted animals was 25 and 28 g, respectively. The ileum was removed and prepared for electrophysiological recording. Either morphine or normorphine (200 - 500 nM) were present in the Krebs solution throughout the electrophysiological studies. The firing rate ( $2.3 \pm 0.3 \text{ Hz}$ ) ( $n = 40$ ), spike waveform and pattern of discharge of these neurons did not markedly differ from those observed in neurons taken from animals not pretreated with morphine ( $3.0 \pm 0.2 \text{ Hz}$ ) (mean  $\pm$  S.E.M.;  $p > 0.05$ , t-test,  $df = 119$ ).

Increasing the concentration of morphine or normorphine up to  $10 \mu\text{M}$  was usually without effect on the firing rate ( $n = 47$ ), indicating the development of tolerance (Fig. 20). At  $3 \mu\text{M}$ , morphine inhibited 6 of 21 cells tested, the mean degree of inhibition of these cells being  $53\% \pm 11$ . At  $5 \mu\text{M}$ , morphine inhibited 3 of 12 cells tested by  $56\% \pm 16$ . At  $10 \mu\text{M}$  morphine was without inhibitory effect on 4 cells to which it was applied. Levorphanol ( $1 - 3 \mu\text{M}$ ) did not inhibit firing ( $n = 8$ ). When the perfusing solution was changed to one which differed only in its lack of morphine, a clear increase in firing was observed in 22 of 30 neurons (Table 4). Typi-

cally, the excitation was moderate (2 - 4 fold increase in firing rate) and lasted throughout the period of perfusion with the morphine-free solution (6 - 60 min).

Neuronal firing rates increased markedly when the perfusing solution was changed to one which contained both naloxone (10 nM - 1  $\mu$ M) and morphine (200 nM) (Fig. 20). Naloxone (200 nM) excited 12 of 17 neurons by  $802\% \pm 84$ , and this value agrees well with that obtained from morphine incubated tissue ( $729\% \pm 145$ ). The excitation was rapid in onset, often reached frequencies of 10 - 20 Hz and usually persisted throughout the duration of the exposure (3 - 12 min). Excitations of equal magnitude could usually (65% of experiments) be produced by repeated applications of the same concentrations of naloxone to a single cell; however, in about one-third of cells such excitations became progressively less (Fig. 21). With longer periods of exposure, the excitations progressively declined during the presence of naloxone. The excitatory effect was stereospecific; that is, it was shared by Mr 2266 (100 nM - 300 nM) but not Mr 2267 (100 nM - 1  $\mu$ M) or (+) -naloxone (100 nM - 1  $\mu$ M).

Table 4. A comparison of the effects of narcotic agonists and antagonists on myenteric neurons which have been incubated (~24 h) in Krebs solutions containing morphine and those removed from morphine pretreated guinea-pigs. An excitation indicates that the firing rate at least doubled. An inhibition indicates that the firing rate fell to at least 75% of the control rate.

RESPONSE		Myenteric Neurons	
		morphine incubated tissues (24 - 36 h)	tissue removed from morphine* pretreated animals (1 - 10 h)
Percent of neurons inhibited by opiates	1 $\leq$ $\mu$ M	0 n=55	0 n=10
	2-30 $\mu$ M	22 n=27	25 n=47
Percent of neurons excited by narcotic (10 nM - 1 $\mu$ M) an- tagonists		79 n=38	70 n=46
Percent of neurons ex- cited by perfusing with an opiate free Krebs solution		62 n=21	73 n=30

\*Ileum removed from morphine pretreated animals was placed in a Krebs solution containing morphine or normorphine (200 - 500 nM).

Figure 20. The effect of normorphine and naloxone on myenteric neurons removed from morphine pretreated guinea-pigs. The tissue was perfused throughout the period of recording in a Krebs solution containing morphine or normorphine (200 nM), except for the periods indicated by the open bars. During the periods indicated by the solid and hatched bars the solution which perfused the tissue contained normorphine and naloxone, respectively. Ordinate: spike frequency (bin width - 10 s). Abscissa: time. (a) The neuronal firing rate was slightly decreased by normorphine (NM) (3  $\mu$ M) but was markedly increased (indicated by the area within the dotted lines) by the (-) isomer of naloxone (100 nM). This excitatory effect was not shared by the (+) isomer of naloxone (100 nM). Legend continued on next page.



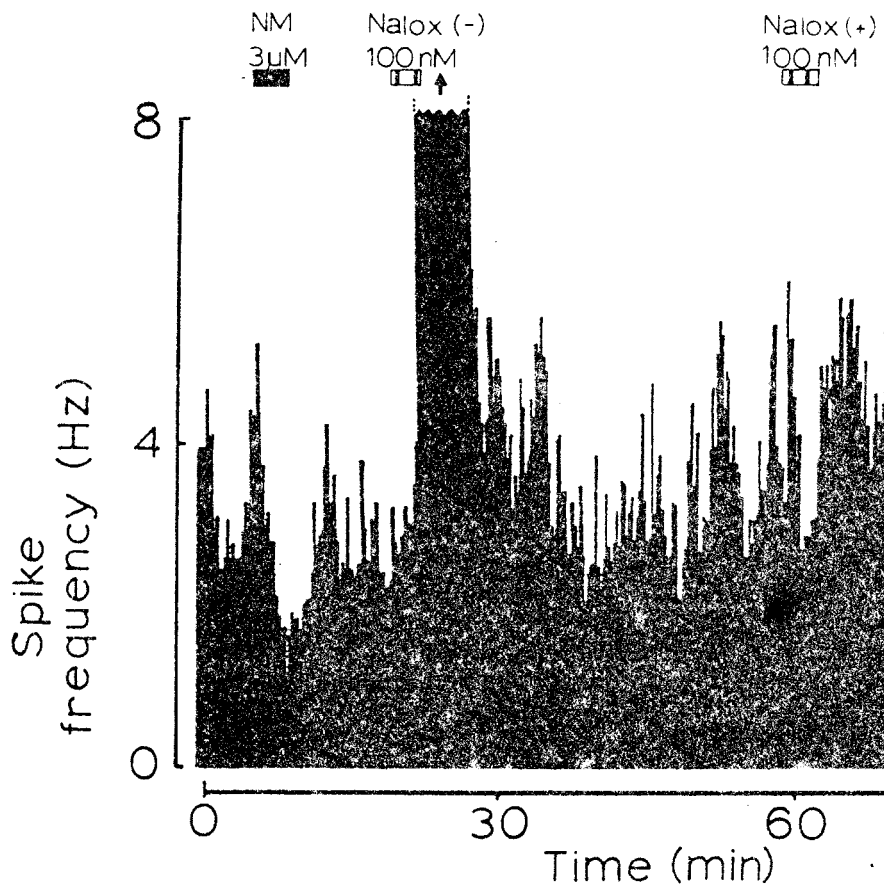


Figure 20b. A different cell from that in (a). The firing rate was increased by removing the morphine from the perfusing solution; and an excitation was also observed when naloxone (100 nM) was added to the bathing solution. The increase in firing produced by naloxone was rapid in onset and 'passed off' during the exposure. Ordinate: spike frequency (bin width - 60 s). Abscissa: time.

Morphine-dependent guinea-pig  
(200 nM NM)

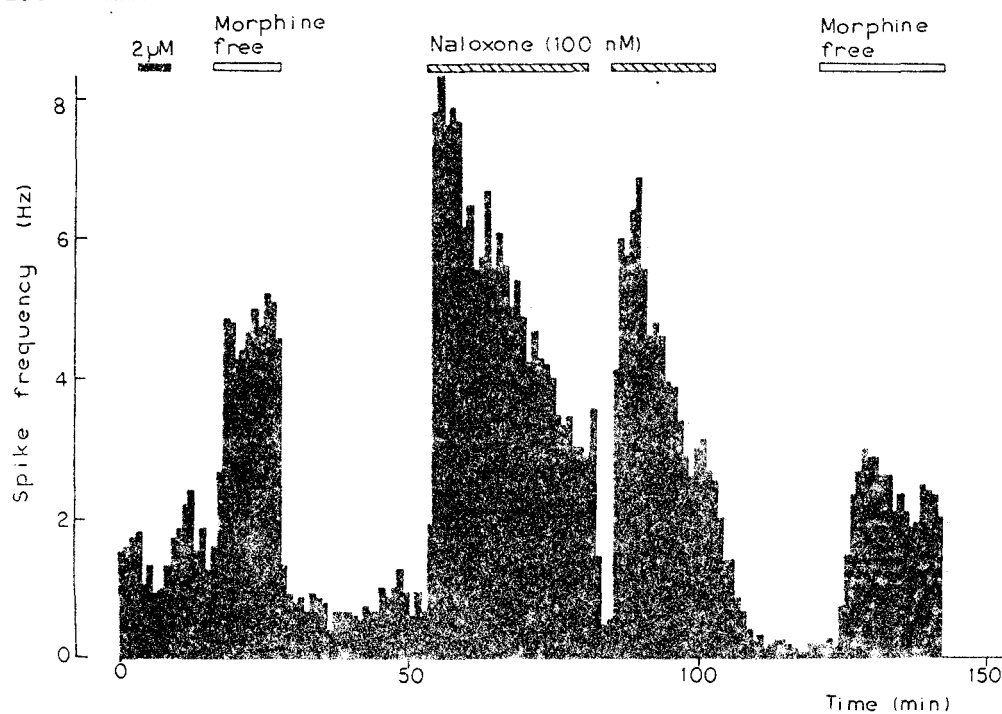
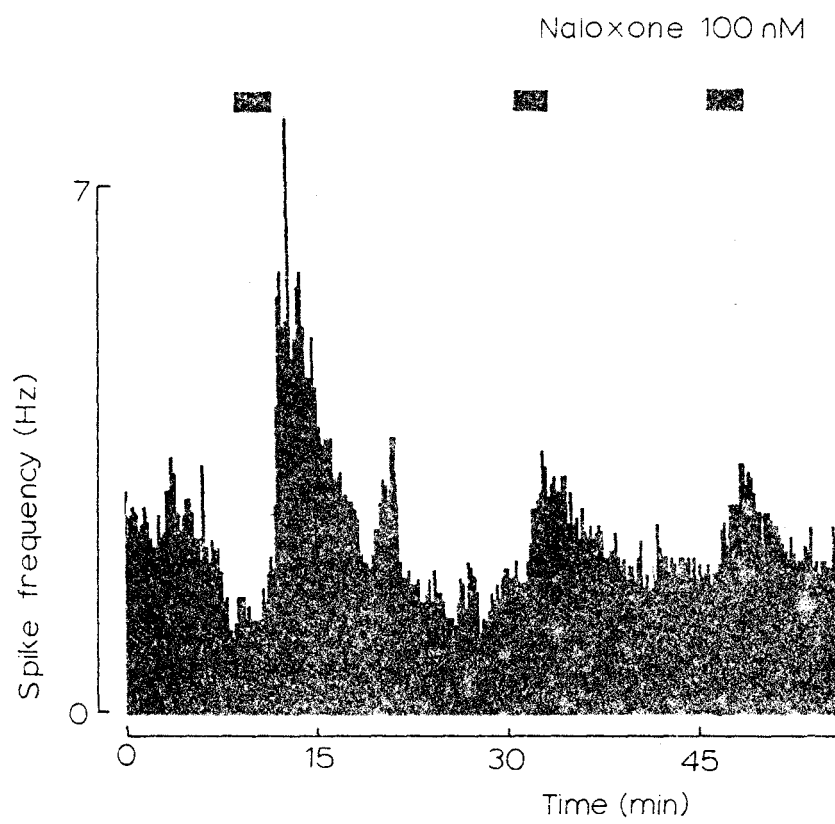


Figure 21. The effect of naloxone on a myenteric neuron removed from a morphine pretreated guinea-pig. The tissue was perfused throughout the period of extracellular recording in a Krebs solution containing morphine (500 nM). Ordinate: spike frequency. Abscissa: time. During the period indicated by the solid bars the solution which perfused the tissue contained naloxone (100 nM). The excitatory response to the same concentration of naloxone (100 nM) diminished with repeated applications.



### 3.6. TIME COURSE OF RECOVERY FROM OPIATE TOLERANCE

#### AND 'DEPENDENCE'

A study was undertaken to determine the time course of recovery from opiate tolerance and dependence. Tissue from morphine pellet implanted guinea-pigs, morphine injected guinea-pigs and from drug naive guinea-pigs which had been incubated in Krebs solution containing morphine ( $1\text{ }\mu\text{M}$ ) for 24 h were studied. In these experiments, morphine was withdrawn from the Krebs solution which perfused the neurons, and at various known intervals thereafter the degree of tolerance, and naloxone induced-excitations (manifestation of dependence; see Methods) was tested. A neuron was described as tolerant if 300 nM morphine (or normorphine) did not inhibit the neuronal firing rate by at least 50%.

Experiments on tissue incubated in morphine for 24 h. The inhibitory effect of a 2 - 3 min application of normorphine (300 nM) was tested at different time intervals after the first exposure of the tissue to a morphine-free solution. A partial return to normal sensitivity (defined as a 50% reduction in firing rate by normorphine 300 nM) occurred rapidly during the first 2 h of morphine withdrawal; 53% of neurons had normal sensitivity at 2 h. However, the proportion of neurons with normal sensitivity rose more slowly thereafter and by 6 - 10 h after morphine removal the percent of cells (70%) which were sensitive was still less than in control conditions (85%) (Fig. 22; Table 5).

Withdrawal of morphine from the bathing solution resulted in an excitation (see section 3.5.2.2.). Following the withdrawal of morphine

Table 5. Time course of the recovery from opiate tolerance in single myenteric neurons. An inhibition indicates that the firing fell to at least 50% of the control rate.

Abbreviations: MFK - morphine-free Krebs solution

## RECOVERY FROM TOLERANCE

I MORPHINE TREATED ANIMALS (IN VIVO)

Hours in MFK	Number of units which have returned to within normal sensitivity*	No. of units tested	Percent
0-1/2	2	8	25
1/2-1	3	10	30
1-2	5	10	50
2-3	5	8	62
3-4	2	3	67
4-6	0	1	0

II PROLONGED INCUBATIONS IN MORPHINE (IN VITRO)

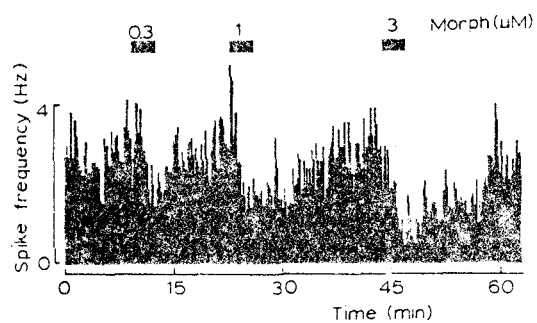
Hours in MFK	Number of units which have returned to within normal sensitivity*	No. of units tested	Percent
0-1/2	0	4	0
1/2-1	1	6	17
1-2	9	17	53
2-3	5	9	56
3-4	2	3	67
4-6	4	6	67
6-10	7	10	70

\*Normal sensitivity was considered to be a 50% inhibition of neuronal firing by normorphine (or morphine) 300 nM.

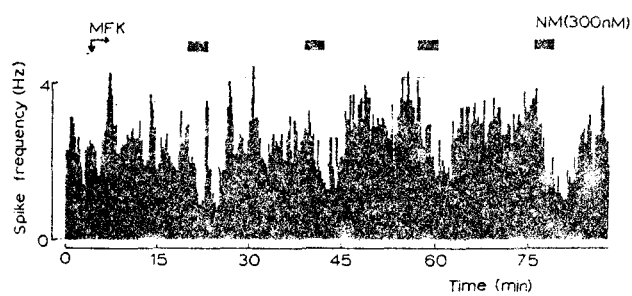


Figure 22. The effect of morphine (or normorphine) on myenteric neurons undergoing opiate withdrawal. Ordinate: spike frequency (bin width - 10 s). Abscissa: time. During the period indicated by the solid bars, morphine (or normorphine) was added to the perfusing solution. (a) This neuron had been incubated in morphine ( $1\text{ }\mu\text{M}$ ) for 24 h and prior to the start of this recording (time zero) had been exposed to a morphine free Krebs (MFK) solution for 8 h. Morphine ( $0.3 - 3\text{ }\mu\text{M}$ ) inhibited the neuronal firing rate in a concentration dependent manner, but the inhibition of firing caused was not so large as in non-incubated tissue (see Figs. 3 & 8). That is, tolerance to morphine's effect was present even after 8 h in MFK. (b) This neuron had been incubated in morphine ( $1\text{ }\mu\text{M}$ ) for 24 h and at the arrow, the perfusing solution was changed to one which was free of morphine. Application of normorphine ( $300\text{ nM}$ ) at intervals of 15 min resulted in a progressively increasing inhibition of firing during the next 1 h. However, even after 1 h,  $300\text{ nM}$  normorphine did not cause maximal inhibition of firing. (c) Tissue was removed from a morphine pretreated guinea-pig and exposed to a Krebs solution containing morphine ( $200\text{ nM}$ ). Thirty min prior to the beginning of the recording (time zero) the solution was changed to one free of morphine. Morphine ( $400\text{ nM} - 4\text{ }\mu\text{M}$ ) only weakly inhibited the firing rate, whereas in control tissue a complete

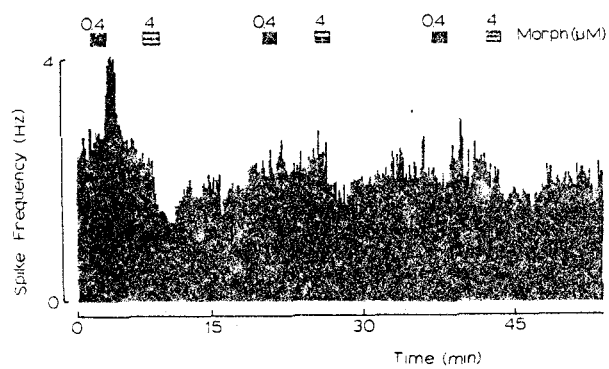
a



b



c



sue removed from morphine treated animals or ileum exposed 24 h to morphine (1  $\mu$ M).

sue removed from morphine treated animals or ileum exposed 24 h to morphine (1  $\mu$ M).

from the perfusing solution, the neurons rapidly lost their propensity to be excited by naloxone. In these experiments naloxone was applied only once (3 min duration) to any neuron, the time of this application being 15, 30, 45 or 60 min after withdrawal of opiate. At 15 min all neurons ( $n = 2$ ) were excited by naloxone; (+) -naloxone was ineffective. The proportion of neurons excited by naloxone fell during the first hour in a morphine-free solution (Table 6) until it was not different from that in tissue from naive animals never exposed to morphine (23%).

Experiments on tissue from morphine pretreated guinea-pigs. Within 2 h of changing to a morphine-free Krebs solution, half of the neurons tested had returned to normal opiate sensitivity (Table 5). In those cells which had recovered their normal sensitivity after withdrawal of morphine, the effect of subsequent prolonged exposure (30 - 60 min) to normorphine was studied. Normorphine (300 nM - 1  $\mu$ M) inhibited the neuronal firing by more than 75%, but in 4 of 7 cells tested this inhibition passed off during the presence of the normorphine (Fig. 23). In 2 of these 4 cells, a marked increase in neuronal firing (frequencies > 20 Hz) was observed upon the washout of the normorphine. Such a decline in the inhibitory effect of normorphine (300 nM or 1  $\mu$ M), or excitations upon washout of normorphine were never observed during experiments on naive tissue. These limited observations suggest that the signs of tolerance and excitations on withdrawal of agonist can be readily reinduced in tissue which had previously had prolonged exposure to opiates.

Tissues were tested with naloxone (100 - 200 nM) at different

periods from the time of morphine withdrawal. A single tissue was exposed to naloxone only once. The degree of excitation caused by naloxone (100 - 200 nM) became less as the time from withdrawal of morphine increased (Fig. 24). The excitations themselves were rapid in onset and offset but rarely lasted throughout the period of exposure (3 - 6 min). The excitation was not mimicked by (+) -naloxone (100 - 200 nM). The proportion of neurons excited by naloxone also declined progressively (Table 6). Sixty-six percent of the neurons were excited by naloxone at 15 min whereas only 25% were excited after 45 min of morphine removal. This latter value is not different from the proportion excited by naloxone in naive tissue and control incubated tissue (see sections 3.2.8. and 3.5.2.1.).

These findings reveal no major differences in the action of opiate agonists and antagonists on guinea-pig myenteric neurons exposed to opiates in vitro or in vivo (Tables 4, 5 & 6).

Table 6. The effect of naloxone on myenteric neurons undergoing withdrawal from morphine. The number of myenteric neurons which showed a naloxone induced excitation decreased with increasing periods of exposure in morphine free Krebs (MFK) solution. An excitation indicates at least a doubling of the firing rate. After 45 min in a MFK solution naloxone failed to produce a significant number of excitations (25%) when compared with control tissues (15-23%).

## TIME COURSE IN THE LOSS OF NALOXONE INDUCED EXCITATIONS

MORPHINE TREATED ANIMALS (IN VIVO)

I

Minutes in MFK	Number of units excited (>2x) by naloxone (100-200 nM)	No. of units tested	Percent
0-15	2	3	66
15-30	2	3	66
30-45	2	4	50
45-60	1	4	25
60-75	0	1	0

PROLONGED INCUBATIONS IN MORPHINE (IN VITRO)

II

Minutes in MFK	Number of units excited (>2x) by naloxone (100-200 nM)	No. of units tested	Percent
0-15	2	2	100
15-30	3	4	75
30-45	1	4	25
45-60	1	4	25
60-75	0	1	0
75	1	5	20



Figure 23. The effect of normorphine on myenteric neurons removed from a morphine pretreated guinea-pig, and subsequently perfused in a morphine free Krebs solution. (a) Before the beginning of this recording (time zero) the neuron had been exposed to a morphine free Krebs (MFK) solution for 105 min. Ordinate: spike frequency (bin width - 10 s). Abscissa: time. The solid bar indicates the period during which the bathing solution was changed to one containing normorphine (NM). Exposure to normorphine (1  $\mu$ M) resulted in an inhibition of neuronal firing which 'passed off' during the presence of the normorphine (contrast this with Figs. 8 & 15). Washout of the normorphine produced an excitation. Legend continues on the following page.

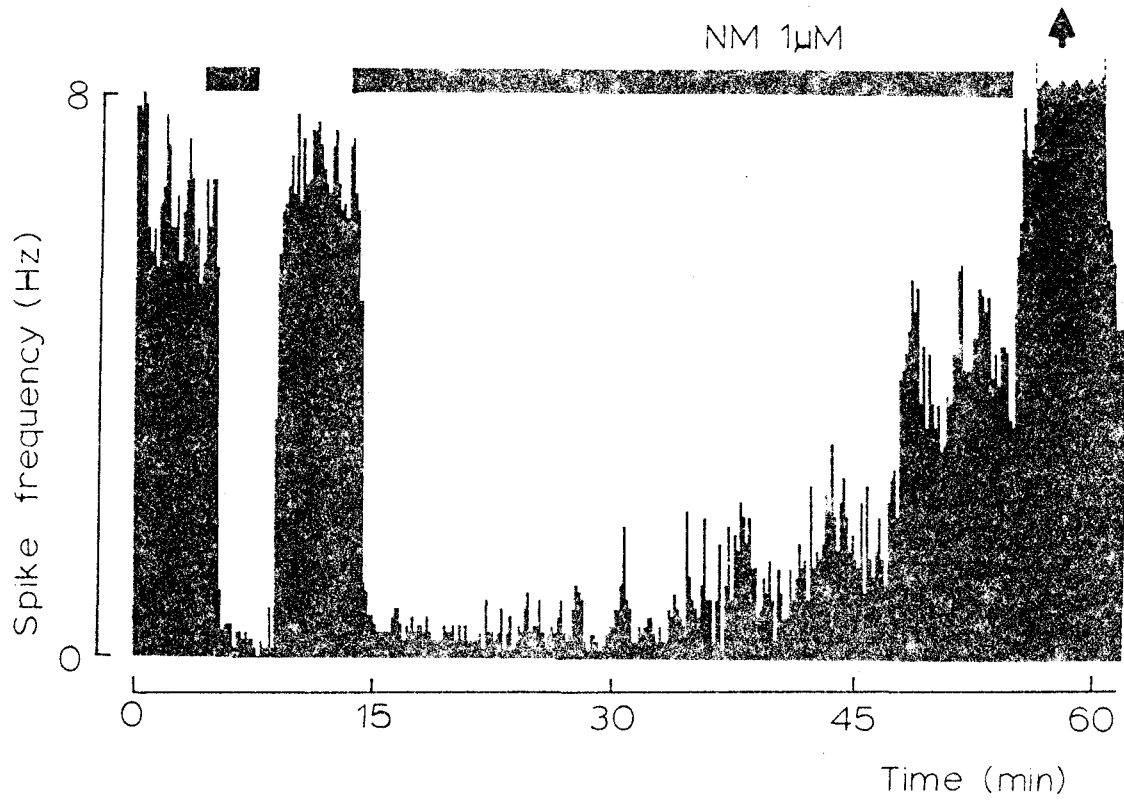


Figure 23b. At time zero, the neuron had been exposed to MFK for 70 min. Normorphine (300 nM) inhibited the firing rate, and with the prolonged exposure to the narcotic 'tachyphylaxis' to the inhibitory effect was observed. Upon washout of the normorphine, no marked increase in neuronal activity was observed. In tissue taken from drug naive guinea-pigs, exposure to normorphine (300 nM - 1  $\mu$ M) for periods of up to 60 min caused an inhibition of firing which persisted throughout the period of application.

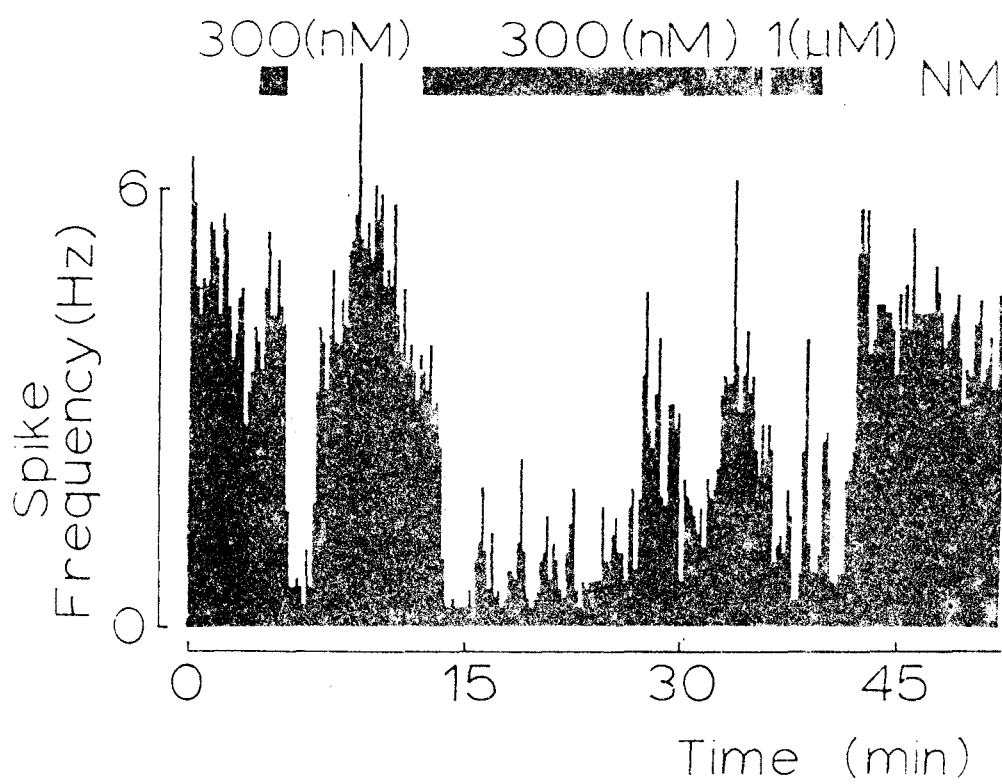
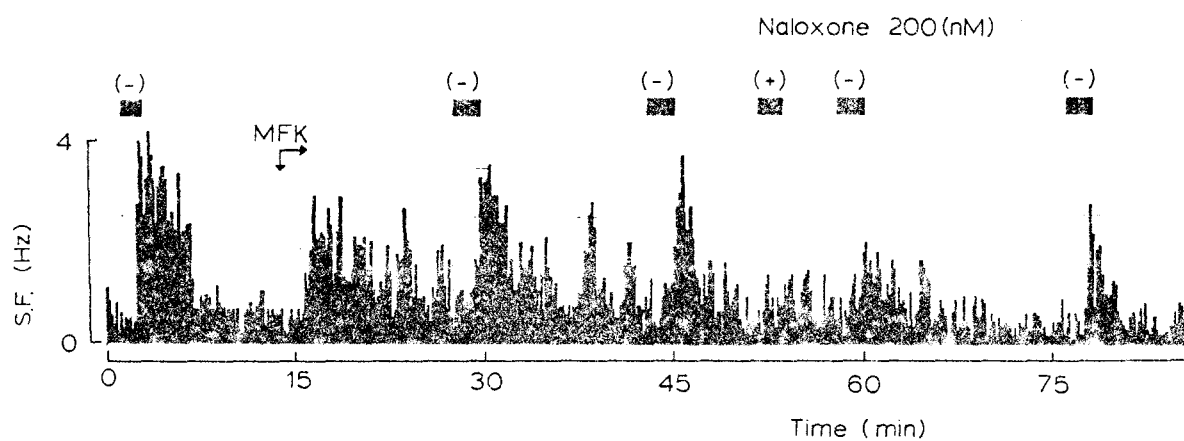


Figure 24. The effect of naloxone on the firing rate of a myenteric neuron removed from a morphine pretreated guinea-pig. Before the beginning of the recording the tissue had been perfused in a Krebs solution containing morphine (200 nM). Ordinate: spike frequency (bin width - 10 s). Abscissa: time. The solid bar indicates the period during which the bathing solution was changed to one containing either the (+) or (-) isomers of naloxone. At the arrow, the perfusing solution was switched to a Krebs solution which was free of morphine (MFK). The excitatory effect of naloxone (-) diminished with each application while naloxone (+) was without effect. The data from this Figure was not included in the results since the tissue was given naloxone more than once.

Morphine dependent animal



### 3.7. THE EFFECT OF SEVERAL AGENTS ON THE DEVELOPMENT OF OPIATE TOLERANCE AND 'DEPENDENCE' AT THE SINGLE NEURON LEVEL

#### 3.7.1. Synaptic Transmission Blockade

##### 3.7.1.1. Hexamethonium and Hyoscine

Incubations in hyoscine and hexamethonium. Tissues were incubated for 24 h in a solution containing hyoscine (1  $\mu$ M) and hexamethonium (300  $\mu$ M). The same solution perfused the tissue during the electrophysiological experiments. The frequency and pattern of neuronal firing was not different from that observed in control incubated tissues. Morphine (300 nM) inhibited the firing of all 6 cells to which it was applied. Naloxone (100 nM - 1  $\mu$ M) had no effect on neuronal firing ( $n = 5$ ). The concentration of hyoscine and hexamethonium used has been shown to block completely cholinergic transmission in the myenteric plexus-longitudinal muscle preparation (Kosterlitz and Lydon, 1971) and to block the fast excitatory postsynaptic potentials recorded from myenteric neurons (Nishi and North, 1973a).

Incubations in morphine, hyoscine and hexamethonium. The effects of opiate agonists and antagonists on tissue incubated for 24 h in a solution containing hexamethonium (300  $\mu$ M), hyoscine (1  $\mu$ M) and morphine (1  $\mu$ M) were studied. The results are based on 20 cells from 7 animals. The tissue was continuously exposed to this solution throughout the period of extracellular recording. The basal firing rate of these cells was  $1.5 \pm 0.3$  Hz and this was not different from the firing rate of tissues incubated in morphine alone ( $1.5 \pm 0.2$  Hz) (mean  $\pm$  S.E.M.).

Increasing the morphine concentration to 10  $\mu$ M was without effect on the firing rate, indicating that tolerance to the inhibitory effect of morphine had developed (Fig. 25a). Levorphanol (1 - 10  $\mu$ M) had no effect on neuronal firing ( $n = 3$ ). When the perfusing solution was changed to one which was free of morphine an increase (411%) in neuronal firing was observed in 2 of 3 cells. Similar but more marked excitations were observed when the solution was changed to one which contained naloxone (in addition to morphine, hyoscine and hexamethonium) ( $n = 16$ ). The excitation produced by naloxone (100 nM) ( $683\% \pm 102$ ) occurred in 11 of 13 neurons and was not distinguishable from the excitation by naloxone ( $636\% \pm 191$ ) in tissue incubated with morphine alone. The excitation was mimicked by Mr 2266 (100 nM - 1  $\mu$ M) ( $n = 3$ ) but not Mr 2267 (100 nM - 1  $\mu$ M) ( $n = 3$ ) or (+) -naloxone (100 nM - 1  $\mu$ M) ( $n = 2$ ) (Fig. 25a; Table 3).

The results described did not differ from those described for tissues incubated in morphine alone (1  $\mu$ M) (see section 3.5.2.2.).

### 3.7.1.2. Lidocaine

Incubations with lidocaine alone. Tissues were incubated for 24 h in a solution containing lidocaine (1 mM). No neuronal activity could be recorded from tissue which continued to be perfused with such a solution. Within 3 - 10 min of changing to a lidocaine-free Krebs solution, neuronal activity could be readily recorded. Extracellular



recordings made in these circumstances were not distinguishable from those made from control incubated tissue; firing rates were unaffected by naloxone (100 nM - 1  $\mu$ M) (n = 7) and inhibited by morphine (300 nM) (Fig. 26). Morphine (300 nM) inhibited 5 of 5 cells and the degree of inhibition was  $86\% \pm 4$  (mean  $\pm$  S.E.M.).

Incubations in morphine and lidocaine. The effects of opiate agonists and antagonists on tissue incubated in morphine (1  $\mu$ M) and lidocaine (1 mM) were tested on 19 units from 5 animals. After the period of incubation, and during the subsequent period of extracellular recording, the Krebs solution contained only morphine (1  $\mu$ M).

The results obtained did not differ from those described above for tissue incubated in morphine alone (see section 3.5.2.2.). Morphine (5  $\mu$ M) inhibited neuronal firing by  $87\% \pm 8$  in 3 of 13 cells tested; 10 cells were unaffected or excited. Naloxone (200 nM) (n = 11) or Mr 2266 (200 nM) (n = 3) produced an immediate and large increase in firing rate ( $829 \pm 116$ ); a less marked excitation (457%) was also observed by changing to a morphine-free solution (3 of 4 cells) (Fig. 25b). The characteristics of the excitation were closely similar to those observed for tissue incubated in morphine alone. Mr 2267 (100 nM - 1  $\mu$ M) and (+)-naloxone (100 nM - 1  $\mu$ M) were without effect on the firing rate (Table 3).

Figure 25. Effect on a myenteric neuron of prolonged exposure to morphine in conditions of synaptic transmission blockade.

Ordinate: spike frequency (bin width - 10 s). Abscissa: time. During the periods indicated by the solid, diagonally hatched, vertically hatched and horizontally hatched bars, the perfusing solution was switched to one which contained morphine, naloxone, Mr (+) and Mr (-), respectively. Note that Mr (-) and Mr (+) refer to the enantiomeric isomers of a benzomorphan (see Methods), Mr 2266 and Mr 2267, respectively. (a) Before beginning the recording (time zero), this neuron had been exposed continuously to a solution containing morphine (1  $\mu$ M), hyoscine (1  $\mu$ M), and hexamethonium (300  $\mu$ M) for 24 h. This solution continued to perfuse the tissue throughout the period of recording except for the time indicated by the open bar, when it contained only hyoscine and hexamethonium. Tolerance to the inhibitory action of morphine was evident in that a very high concentration of morphine (10  $\mu$ M) no longer depressed the neuronal firing rate. Naloxone (100 nM) caused a pronounced increase in the firing (in excess of 10 Hz), Mr 2266 also caused a marked excitation, whereas its (+) isomer, Mr 2267 had no clear effect on the firing rate. Removing the morphine from the perfusing solution resulted in an increase in firing. Legend continued on the following page.

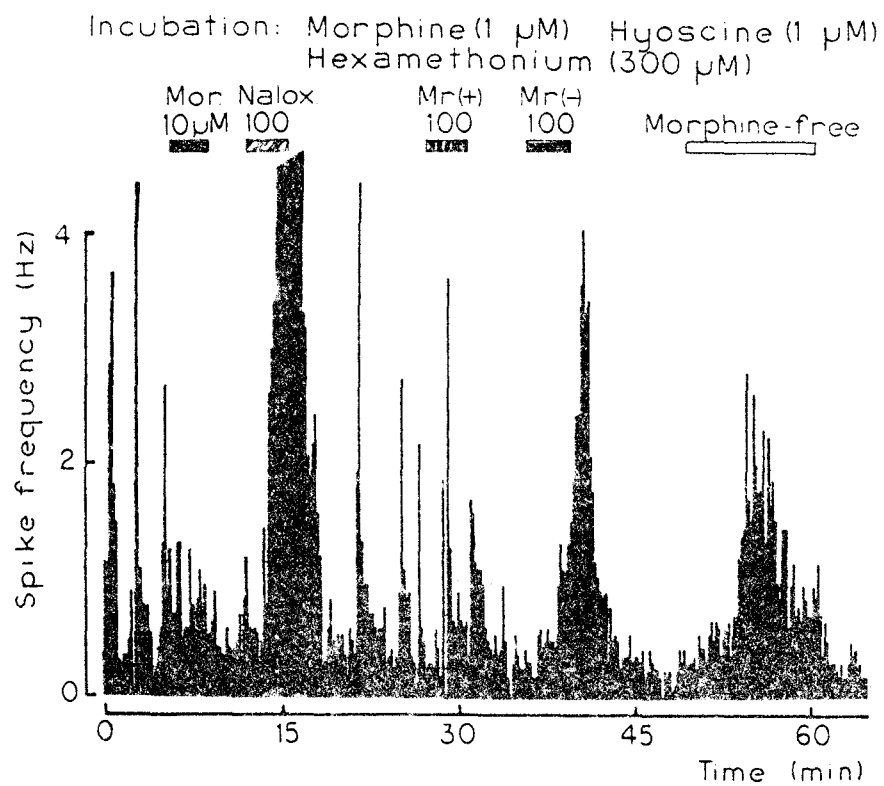


Figure 25b. This neuron had been exposed continuously to a solution containing morphine ( $1\ \mu\text{M}$ ) and lidocaine ( $1\ \text{mM}$ ) for 24 h. Five min prior to the beginning of this recording (time zero), the perfusing solution was changed to one which differed only in its lack of lidocaine and this solution continued to perfuse the tissue throughout the period of recording except for the time indicated by the open bar. Tolerance to the inhibitory effect of morphine had developed, in that morphine ( $5\ \mu\text{M}$ ) did not inhibit the firing rate. Naloxone ( $200\ \text{nM}$ ) and Mr (-) ( $200\ \text{nM}$ ) caused a marked increase in firing (in excess of 10 Hz as indicated by the area within the broken lines), but Mr (+) was without effect. Changing the bathing solution to one which was free of morphine (indicated by open bar) also caused an increase in firing. This increase did not persist throughout the period of exposure.

Incubated in Morphine (1  $\mu$ M), Lidocaine (1mM)

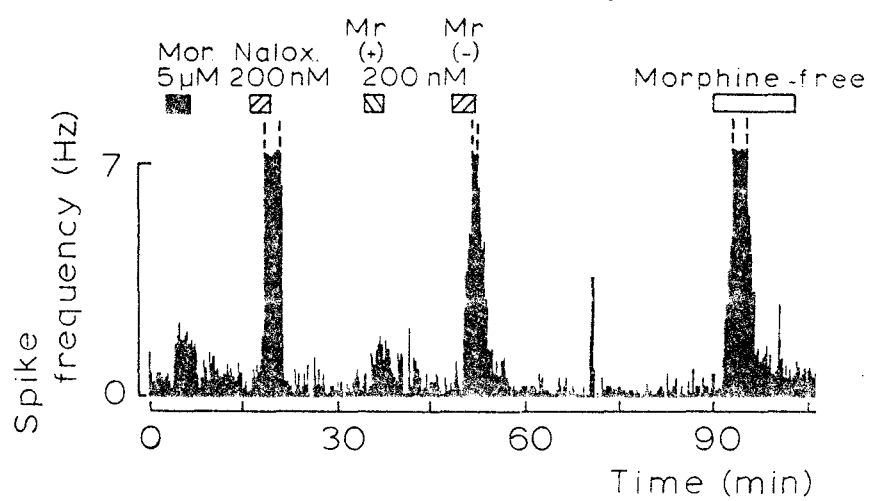
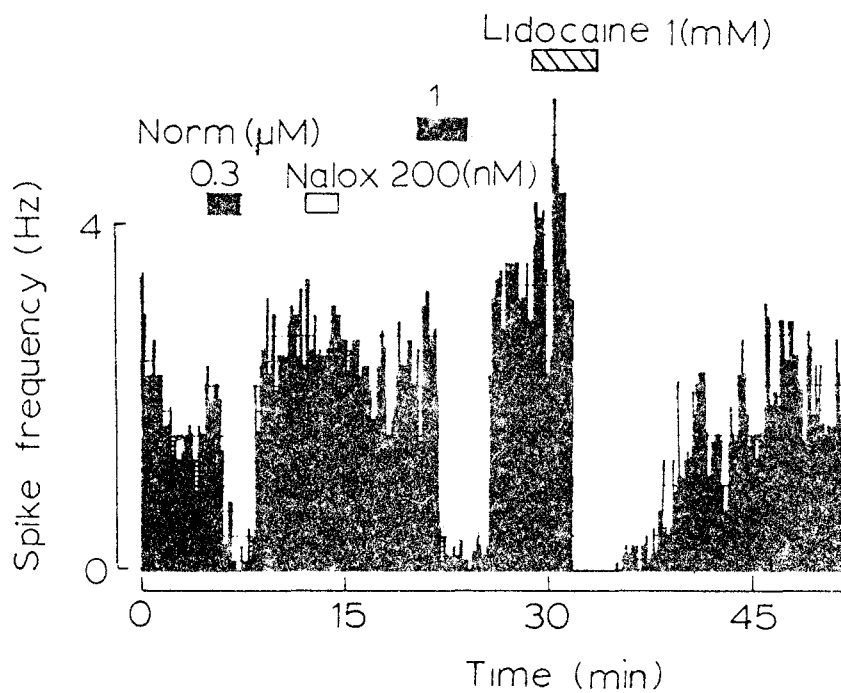


Figure 26. The effect on a myenteric neuron of a prolonged exposure to lidocaine. This neuron had been exposed continuously to a solution containing lidocaine (1 mM) for 24 h. Ten minutes prior to the beginning of the recording (time zero), the bathing solution was switched to one which was free of lidocaine (normal Krehs solution). Ordinate: spike frequency (bin width - 10 s). Abscissa: time. During the periods indicated by the solid, open, and hatched bars, the perfusing solution was changed to one which contained normorphine, naloxone and lidocaine respectively. Normorphine (300 nM - 1  $\mu$ M) caused a dose dependent inhibition of neuronal firing. Naloxone (200 nM) was without effect on the firing rate. Lidocaine (1 mM) depressed neuronal firing in a reversible manner. Compared with control treated tissue, no change in neuronal sensitivity to the agents listed above was observed.

Incubated in Lidocaine



### 3.7.2. Protein Synthesis Inhibitors

Incubations with cycloheximide alone. Tissues were incubated for 24 h in a solution containing cycloheximide (100  $\mu$ M). The concentration of cycloheximide used was similar to the concentration necessary to block the synthesis of enkephalin in the myenteric plexus (McKnight et al., 1978). This solution perfused the tissue throughout the electrophysiological experiments. The rate of discharge and pattern of neuronal firing were not different from those observed in control incubated tissue (see section 3.5.2.1.). Naloxone (100 nM - 1  $\mu$ M) had no effect on neuronal firing ( $n = 6$ ) and morphine (300 nM) inhibited ( $87\% \pm 3$ ) the firing of 7 of 7 cells.

Incubations with morphine and cycloheximide. The effects of opiate agonists and antagonists on tissue incubated in a solution containing morphine (1  $\mu$ M) and cycloheximide (100  $\mu$ M) were tested on 20 cells from 6 animals. The same solution perfused the tissue during the electrophysiological recording. Neuronal firing rates ( $1.4 \pm 0.2$  Hz) ( $n = 11$ ) were not different from those observed in tissue incubated in morphine alone ( $1.5 \pm 0.2$  Hz). In only one of 11 cells, was the firing rate inhibited by morphine (5  $\mu$ M) (Table 3). That is, tolerance existed which was similar to that caused by incubations with morphine alone. Sixty percent of neurons were excited by changing to a solution which contained naloxone (100 nM - 200 nM) in addition to morphine and cycloheximide (Table 3). The excitation caused by naloxone (200 nM) was  $463\% \pm 126$  (5 of 8 cells were excited; 3 were not affected) and this



is not different than that observed with tissue incubated in morphine alone ( $729\% \pm 145$ ) (mean  $\pm$  S.E.M.) ( $p > 0.05$ , t-test,  $df = 11$ ).

### 3.7.3. Cyclic Nucleotides

#### 3.7.3.1. IBMX and Dibutyryl Cyclic-AMP

Incubations with IBMX and dbc-AMP. Tissues were incubated for 24 h in a solution containing IBMX (500  $\mu$ M) and dbc-AMP (200  $\mu$ M); and this solution also perfused the tissue during the electrophysiological experiments. The frequency of neuronal firing ( $1.7 \pm 0.5$  Hz) was reduced when compared with control incubated tissues ( $2.9 \pm 0.4$  Hz). Morphine (300 nM) (or normorphine) inhibited the firing of 7 cells to which it was applied ( $88\% \pm 2$ ). Naloxone (200 nM) had no effect on neuronal firing ( $n = 6$ ).

Incubations with morphine, IBMX and dbc-AMP. The effects were studied of opiate agonists and antagonists on tissue incubated for 24 h in a solution containing morphine (1  $\mu$ M), IBMX (500  $\mu$ M) and dbc-AMP (200  $\mu$ M). This same solution perfused the preparation throughout the subsequent period of extracellular recording. The following results are based on extracellular recordings from 21 neurons from 5 animals. Neuronal firing rates ( $1.6 \pm 0.3$  Hz) were not different from those observed in tissues incubated in morphine alone ( $1.5 \pm 0.2$  Hz) or in IBMX and dbc-AMP ( $1.7 \pm 0.5$  Hz) ( $p > 0.05$ , t-test). Morphine (3 - 10  $\mu$ M) failed to inhibit the firing of 9 of 11 neurons tested; two

neurons were inhibited. Naloxone (200 nM) produced an immediate increase in the firing rate in 9 of 18 neurons ( $557\% \pm 73$ ). The excitation caused by naloxone persisted throughout the period of application (3 min). Mr 2267 (200 nM) ( $n = 3$ ) and (+) -naloxone (200 nM) ( $n = 3$ ) were without effect on the firing rate (Table 3).

#### 4. DISCUSSION

##### 4.1. ACUTE ACTIONS OF OPIATES ON THE SPONTANEOUS ACTIVITY OF SINGLE MYENTERIC NEURONS

Morphine has been shown to inhibit the spontaneous activity of single myenteric neurons recorded extracellularly (Sato et al., 1973; 1974; Dingledine et al., 1974; 1975; 1976; North and Williams, 1977). The inhibition was stereospecific, occurred with low concentrations of morphine and was antagonized by naloxone. The present investigation confirmed these findings with respect to the actions of morphine on spontaneously firing myenteric neurons and was extended to include the study of the acute actions of ketocyclazocine, cyclazocine and buprenorphine.

The inhibition of firing produced by ketocyclazocine and cyclazocine was marked, concentration-dependent, slow in offset and poorly antagonized by naloxone. The effective concentration range of ketocyclazocine and cyclazocine used in the studies of nerve-mediated contractile responses differed from that effective in the extracellular studies. The concentration of ketocyclazocine or cyclazocine required to inhibit the nerve mediated contractile response of the guinea-pig ileum by 50% was less than 4 nM (see section 1.4.3.) whereas the concentration necessary to inhibit the spontaneous activity of single myenteric neurons by 80% was at least 8 fold higher. It is possible that ketocyclazocine (or cyclazocine) may act on receptors different to those affected by morphine (or normorphine) in mediating the inhi-

bition of the contractile response and the inhibition of neuronal firing. Against this possibility is the finding that both actions are blocked by naloxone: however, it is not possible to say on the basis of the present findings whether the same concentration of naloxone ( $\text{pA}_2$ ) is effective against both these agonist effects.

Another possibility is that inhibition of the contractile response is a more sensitive measure of receptor occupancy and that the inhibition of neuronal firing requires a relatively higher occupancy before its effects are apparent with the present experimental techniques. Against this possibility is the finding that the  $\text{IC}_{50}$ 's for morphine, normorphine and enkephalin in inhibiting the contractile response of the guinea-pig ileum (Waterfield, Smokcum, Hughes, Kosterlitz and Henderson, 1977) agree well with the concentration of these agonists required to inhibit cell firing by approximately 50% (present results; also North and Williams, 1977; Williams and North, 1979a).

The effect of buprenorphine on the spontaneous firing rate of single myenteric neurons was quite variable (30% of the neurons tested were excited, 23% were inhibited and 47% were not affected). The fact that buprenorphine was without inhibitory effect on 77% of neurons studied was somewhat surprising since all other narcotics tested inhibited 70-80% of the myenteric neurons to which they were applied. Buprenorphine is a highly lipophilic narcotic analgesic with an extremely slow rate of onset and offset (several hours) in the isolated guinea-pig ileum (Kosterlitz et al., 1975; Schulz and Herz, 1976b). Because of the spontaneous changes in firing rate of individual neurons with time and because successful recordings from neurons usually lasted only 1-2 h, drug effects which are of slow onset would be difficult to detect. In addition, with the present technique it is difficult to ascribe any

action to a drug if it cannot be reversed by washing. On the basis of previous studies (Schulz and Herz, 1976b), it would not be expected that buprenorphine's effect on spontaneously firing neurons would rapidly washout. The basis of the excitatory action of buprenorphine was not fully investigated: it differed from the inhibitory effect in being reversible on washing. This argues against the possibility that the small proportion of neurons inhibited by buprenorphine was the result of a concomitant excitation mediated through a different mechanism.

Analysis of the binding properties of buprenorphine in rat brain homogenates has revealed the presence of saturable binding sites similar to those demonstrated by other workers for a series of opiates (Hambrook and Rance, 1976). In this binding assay, buprenorphine had a 'sodium-shift' typical of narcotic antagonists. In the present electrophysiological experiments, myenteric neurons were no longer sensitive to the inhibitory effects of normorphine at any time following the administration of buprenorphine. That is, buprenorphine acted in a manner which was similar to naloxone (see section 4.3.2.). This is the first demonstration of antagonist properties of buprenorphine at the single neuron level (Fig. 7).

Do multiple forms of the opiate receptor exist on single neurons of the myenteric plexus? One purpose of studying the effects of morphine, ketocyclazocine and cyclazocine on spontaneously firing myenteric neurons was to test the hypothesis that some neurons would respond to only one of these prototypic receptor subtype agonists. The firing rate of single myenteric neurons was inhibited by morphine ( $\mu$  &  $\kappa$  agonist), cyclazocine ( $\sigma$  &  $\kappa$  agonist), ketocyclazocine ( $\kappa$ -agonist) and enkephalin ( $\delta$ -agonist). In many experiments, several of these agonists were

effective on the same neuron. This implies that if these agonists are really selective for the proposed receptor subtypes (Martin et al., 1976) then all subtypes exist on each neuron. The restriction of the range of concentrations of cyclazocine and ketocyclazocine which were tested in the present study imposes some limits on the interpretation of the findings with respect to any such receptor subtype selectivity; however, the present findings, and previous ones with enkephalin, suggest that neurons do not bear only one receptor subtype.

## 4.2. THE INVOLVEMENT OF CYCLIC AMP IN THE ACUTE INHIBITORY ACTION OF OPIATES ON NEURONAL FIRING

Morphine has two actions in the myenteric plexus of the guinea-pig ileum; namely, inhibition of neuronal firing (Sato et al., 1973) and inhibition of neurotransmitter release (Paton, 1957). The principal aim of the present experiment was to test the hypothesis that inhibition of adenylate cyclase (and the resultant fall in cyclic-AMP levels) is an intermediate step between occupation of the opiate receptor by the narcotic agonist and the inhibition of neuronal firing which it produces. The evidence for this hypothesis has been given in section 1.3.

### 4.2.1. Inhibition of Neuronal Firing

The fact that both cyclic-AMP and morphine have similar effects on myenteric neurons has led to certain difficulties for theories suggesting an inhibition of adenylate cyclase activity as the underlying mechanism of opiate action. Furthermore, the finding that the inhibition of neuronal firing by cyclic-AMP was antagonized by theophylline does not support the intracellular site of action for this compound which would be expected if cyclic nucleotides were to mediate the acute actions of opiates. Similar findings at other sites have led investi-

gators to conclude that the inhibition caused by the adenine nucleotides is mediated through activation of an extracellular receptor (Nathanson, 1977; Hiyashi et al., 1978). Compatible with an extracellular site of drug action for these compounds was the finding that cyclic-AMP was more effective in inhibiting neuronal activity than its dibutyryl derivative, which supposedly penetrates through cell membranes more easily (Sawynok Jhamandas, 1976).

If we assume that morphine inhibits cyclic-AMP formation in the myenteric plexus of the guinea-pig, and that this is responsible for the inhibition of neuronal firing which morphine produces, then attenuation of the effect on cell firing might be expected in conditions where levels of cyclic-AMP are elevated. However, no difference in the effectiveness of morphine was found under such conditions. Prior or concurrent administration of dibutyryl cyclic-AMP (or cyclic-AMP) did not reduce the sensitivity of the tissue to the opiates (Fig. 11). The adenine nucleotides appear to inhibit cell firing themselves by acting on the outer surface of the cell membrane; if a substantial amount enters the cell, as is likely in the case of dbcyclic-AMP, then this is ineffective in modulating opiate action.

The results obtained with prostaglandin  $E_2$  and IBMX likewise do not support the hypothesis that inhibition of adenylate cyclase is an intermediate step in the acute action of opiates. The sensitivity of the tissue to the opiates was not altered by pretreatment with  $PGE_2$  or IBMX. Prostaglandin  $E_2$  (10 - 30 nM) alone often had a transient excitatory effect on neuronal firing, and higher concentrations led to marked excitations of neurons and a direct excitation of the longitudinal muscle. The concentration of  $PGE_2$  necessary to cause a 2.7 fold increase



in cyclic-AMP levels in rat brain homogenates was 90  $\mu\text{M}$  (Collier and Roy, 1974), and the concentration required to stimulate adenylate cyclase in cultured neuroblastoma-glioma was 10  $\mu\text{M}$  (Sharma, Klee and Nirenberg, 1975). The possibility exists, therefore, that a 1000 fold increase in prostaglandin  $\text{E}_2$  may have revealed an antagonism of the neuronal inhibitory action of morphine. A similar criticism does not apply to the experiments with IBMX. In this case a concentration of 50  $\mu\text{M}$  was used which is 10 times the  $\text{IC}_{50}$  for inhibition of cyclic-AMP phosphodiesterase in isolated fat cells (Beavo, Rogers, Croffard, Hardman, Sutherland and Newman, 1970) and 2 times the  $\text{IC}_{50}$  for phosphodiesterase inhibition in rat brain homogenate (Francis, Cuthberg, Saeed, Butt and Collier, 1978).

The present results are in general agreement with those reported by Duggan and Griersmith (1979) for cat spinal neurons. These investigators did not observe an effect of electrophoretically applied cyclic-AMP, its butyryl derivatives or IBMX on the nociceptive responses of neurons located within lamina IV and V of the spinal cord nor did these substances alter the ability of electrophoretically applied morphine to depress the response to noxious stimulation.

#### 4.2.2. Inhibition of Acetylcholine Release

Adenosine and related nucleotides depress the nerve mediated contractile response of the longitudinal muscle of the guinea-pig ileum by inhibiting the release of acetylcholine (Takagi and Takayanagi, 1972;

Gintzler and Mussachio, 1975; Sawynok and Jhamandas, 1976; Hayashi et al., 1978). This action is similar to that reported for morphine on the guinea-pig isolated ileum (Paton, 1957). However, the mechanism of action whereby these compounds are able to depress the release of acetylcholine from the cholinergic motoneurons appears to be different (see section 1.3.4.).

Inhibition of the electrically induced contraction of the isolated guinea-pig ileum by morphine is antagonized by prostaglandin  $E_1$  or  $E_2$  (Schulz and Cartwright, 1976; Ehrenpreis, Greenberg and Belman, 1973). It is important to stress that this antagonism is not specific for opiate agonists since the inhibitory action of non-opiates, such as, tetrodotoxin and adrenaline were also effectively antagonized by  $PGE_1$  (Schulz and Cartwright, 1976). This action of PGE does not appear to be related to its ability to stimulate adenylate cyclase but rather to sensitize the smooth muscle to acetylcholine and in part to increase the release of acetylcholine from the myenteric plexus (Schulz and Cartwright, 1976).

#### 4.2.3. Conclusion

The present results do not support the hypothesis that the inhibition of adenylate cyclase is relevant to the acute inhibitory action of morphine on single mammalian neurons. However, it is possible that activation of only the  $\delta$ -receptor subtype might be linked to the inhibi-

tion of adenylate cyclase activity, because the cultured neuroblastoma cells possess only  $\delta$ -receptors (Chang et al., 1979). The studies on neuroblastoma-glioma hybrid cells provide by far the strongest evidence for an involvement between opiates (or opioid peptides) and cyclic nucleotides. However, North and Vitek (1980) were unable to demonstrate any interaction between either a specific  $\delta$ -receptor agonist or  $\mu$ -receptor agonist and the cyclic nucleotides when they studied the ability of their agents to depress the excitatory junction potential recorded intracellularly from the mouse vas deferens. That is, the  $IC_{50}$ 's for D-Ala<sup>2</sup>-D-Leu<sup>5</sup>-enkephalin (DADL) ( $\delta$ -receptor agonist) and normorphine ( $\mu$ -receptor agonist) were unchanged in the presence of dbcyclic-AMP and IBMX.

It has not been demonstrated that opiates inhibit adenylate cyclase activity in the myenteric plexus of the guinea-pig. If they do, then this is probably not an intermediate step in the acute inhibition of neuronal firing which they produce. However, it is possible that cyclic-AMP involvement may distinguish the acute from the long term actions of opiates in the guinea-pig myenteric plexus (see below) (Collier, 1980).

#### 4.3. PROLONGED EXPOSURE TO OPIATES IN VIVO OR IN VITRO

Extracellular recordings were made from myenteric neurons either removed from morphine pretreated guinea-pigs, or incubated in opiate containing Krebs solution. The effect of opiate agonist and antagonists on these neurons were compared with their effect on myenteric neurons from drug naive guinea-pigs. The present results have extended previous findings on tolerance and 'dependence' demonstrated in whole ileum or myenteric plexus longitudinal muscle (MPLM) preparations to the level of the single neuron.

The four major questions addressed by the present study are as follows. First, is the reduced inhibitory effect of morphine on neuronal firing and the marked excitation of neurons caused by naloxone indicative of opiate tolerance and dependence? Second, does the recovery from opiate tolerance and 'dependence' in single neurons parallel the time course of recovery in MPLM preparations or even whole animals? Third, is the tolerance and 'dependence' demonstrated in myenteric neurons following in vivo exposure to morphine similar to that produced by in vitro exposure to morphine? Fourth, can drugs incubated concomitantly with morphine modify any development of tolerance and 'dependence' which is produced? These points will be discussed in order.

#### 4.3.1. Tolerance to the Effect of Morphine

Prolonged exposures to morphine in vivo or in vitro diminished the effectiveness of subsequent applications of morphine in inhibiting the spontaneous firing rate of myenteric neurons. Three findings indicate the development of tolerance to opiates. First, spontaneous firing of neurons could be readily recorded even when the bathing solution contained morphine at a concentration which normally inhibited completely the firing rate of all opiate sensitive neurons. Second, increasing the concentration of morphine to 5 or 10  $\mu\text{M}$  was without any inhibitory effect on approximately 80% of the neurons. Third, levorphanol and met-enkephalin had no effect on the firing rate of cells pretreated with morphine: on the other hand, cyclic-AMP had the same inhibitory effect on morphine pretreated neurons as on untreated cells.

Opiate tolerance induced in single myenteric neurons in vitro may not have been complete after 24-36 h of incubation. The mean firing rate of neurons incubated with morphine was lower ( $1.5 \pm 0.2$  Hz) (mean  $\pm$  S.E.M.) than that of control incubated tissue ( $2.9 \pm 0.4$  Hz); there was a significant difference between the mean firing rates of the two groups ( $p < 0.05$ , t-test,  $df = 44$ ). With such a large variability of firing rates within each group, any difference in frequency would have to be large, or a much larger sample would be required, in order for it to be detected. The mean basal firing rate of neurons from ileum removed from morphine pretreated guinea-pigs ( $2.4 \pm 0.2$  Hz) (mean  $\pm$  S.E.M.) was not different from that of freshly removed tissue ( $3.0 \pm$

0.2 Hz) thus indicating that tolerance to morphine exposure in vivo was probably complete ( $p > 0.05$ , Student's t-test,  $df = 19$ ).

#### 4.3.2. Manifestation of 'Dependence' -- Effect of Naloxone

Addition of naloxone to tissue chronically exposed to morphine either in vitro or in vivo resulted in an increase in neuronal activity. This excitation almost certainly reflects at the cellular level the sustained contracture seen in the twitch studies (Schulz and Herz, 1976a). Naloxone caused an excitation in 79% of the myenteric neurons exposed to morphine in vitro and 70% of the neurons exposed to morphine in vivo. This neuronal excitation was not simply a reversal of inhibition due to the morphine present in the bathing solution for the following reasons. First, the frequency of firing produced by naloxone often exceeded 10-20 Hz, a discharge rarely attained in normal tissue. Second, repeated exposures to naloxone resulted in progressively smaller excitations. It is possible that with repeated applications of naloxone the tissue has become less dependent on morphine and, thus, exhibits weaker excitations (signs of dependence) to subsequent naloxone challenges. An excitatory effect of naloxone due to a reversal of the inhibitory action of morphine present in the bathing solution would be expected to be constant with each application. Third, prolonged exposures (greater than 60 minutes) to naloxone resulted in the neuronal excitation 'passing off'

during the presence of naloxone. This finding could also be explained by a progressively reduced dependence on morphine. The excitation was mimicked by the benzomorphan antagonist (Mr 2266) but not its inactive (+)-isomer (Mr 2267) or the (+)-isomer of naloxone. This stereospecificity indicates that the excitation is probably mediated through an action on the opiate receptor. A less intense excitation was observed by changing the bathing solution from one which contained opiate to one which was free of opiate.

Is it possible that it was the morphine present in the bathing solution, (which was added to maintain any changes induced by the prolonged in vivo or in vitro exposure to morphine) and not the long term exposure to morphine itself which was responsible for the changes in sensitivity to opiate agonists and antagonists that were observed? Some evidence has been presented already against the possibility that it is an ongoing inhibitory action of the morphine which is responsible for these changes; for example, the finding that repeated applications of naloxone do not cause repeated neuronal excitations (see above). Furthermore, application of normorphine (1  $\mu$ M) to naive tissue for periods of 60 min caused an inhibition of firing which persisted throughout this period, and naloxone did not cause excitations (other than simple reversal of morphine depression) when applied after 60 min exposure to normorphine (or morphine). Therefore, the possibility that the presence of morphine in the bathing solution causes a rapid desensitization of the tissue is unlikely on the basis of these two findings and because high concentrations of morphine still produced an inhibition of firing.

### Tolerance and 'Dependence'

Tolerance and 'dependence' was induced by prolonged exposures to levorphanol or ketocyclazocine but not by prolonged exposures to dextrorphan. Levorphanol incubations induced tolerance to the effects of both morphine and levorphanol whereas dextrorphan incubations induced tolerance to neither. Ketocyclazocine incubations induced tolerance to the effect of ketocyclazocine but demonstrated poor cross-tolerance to the effects of morphine or normorphine (Fig. 19). Naloxone appeared to be less effective in precipitating a withdrawal reaction (excitation) in tissues incubated with ketocyclazocine as compared to tissues incubated with morphine or levorphanol.

Morphine failed to inhibit the firing rate of myenteric neurons incubated in buprenorphine, but more importantly, naloxone failed to excite these same neurons. It is not clear whether failure of morphine to inhibit the firing rate of these neurons represents the development of cross-tolerance which exists amongst opiates or simply an ongoing antagonist action of buprenorphine (see section 4.1.). In support of the latter possibility was the finding that buprenorphine antagonized the antinociceptive actions of morphine in the mouse and rat tail flick tests, precipitated signs of abstinence in morphine dependent mice (actions which are analogous to those seen with narcotic antagonists) (Cowan et al., 1977) and antagonized the inhibitory effect of morphine on the spontaneous activity of single myenteric neurons (present study).

Even if buprenorphine induces the underlying changes of tolerance and dependence, naloxone may be unable to excite these cells because of the high affinity of the opiate receptor for buprenorphine (which exceeds that for naloxone) and/or the high lipid solubility



of buprenorphine (Hambrook and Rance, 1976; Rance and Dickens, 1978). The present findings on the long term effects of buprenorphine are in agreement with those established for the guinea-pig isolated ileum (Schulz and Herz, 1976b), monkey (Cowan et al., 1977) and human (Jasinski et al., 1978). The conclusion which is reached from these studies is that buprenorphine is an opiate agonist with a low capacity to induce drug dependence.

These effects of opiate agonists and antagonists on myenteric neurons incubated in opiate containing Krebs solution are in many ways analogous to the behavioral effects observed in humans or animals subjected to long term administration of morphine, levorphanol, buprenorphine or ketocyclazocine (see section 1.1.3.).

The phenomena of opiate tolerance and dependence have long been considered inseparable. However, the present findings with long term dextrorphan incubations support a possible dissociation of these two events. Tissues incubated in dextrorphan ( $1\text{ }\mu\text{M}$ ) were still sensitive to the inhibitory action of morphine (absence of cross-tolerance) but when naloxone was added to the perfusing solution or when dextrorphan was removed a marked excitation occurred which was similar to that observed with morphine incubations (manifestation of 'dependence'). This action of naloxone was not observed with incubations at the lower concentration of dextrorphan ( $100\text{ nM}$ ). It is possible that dextrorphan acts as a weak opiate agonist at the higher concentration (Kosterlitz and Waterfield, 1975b) and incubation of tissue with this drug induces aspects of opiate dependence without evidence of tolerance. A dissociation of tolerance from physical dependence has been previously reported for alcohol (Ritzmann and Tabakoff, 1976), LSD (Jaffe, 1975) and caffeine

(Ritchie, 1975). An alternative explanation is that the two phenomena are inextricably linked but have different time courses in their development. That is, the development of opiate tolerance requires a longer period of time than the development of opiate dependence. If the conditions of the incubation could be modified so as to maintain viability of the preparation for several days in vitro -- then the effects of opiate agonists and antagonists on myenteric neurons exposed to dextrorphan for different lengths of time could be studied. In this way it might be possible to determine whether the two events are separable or just have different time courses in development.

#### 4.3.3. Time Course of the Recovery from Opiate Tolerance and 'Dependence'

Extracellular recording from single myenteric neurons from ileum chronically exposed to morphine in vitro or in vivo were made. Within 2 h of switching the bathing solution from one which contained morphine to one which was similar but lacked morphine, one-half of the neurons tested had returned to normal opiate sensitivity (in vitro incubations 53%; in vivo incubations 50%). A second much slower component of the recovery process followed (Table 5). The absence of any inhibition by morphine (300 nM) or an inhibition which reduced the firing rate to not less than 50% of the control rate were the criteria used to establish a cell as tolerant. The question which might then

be asked is whether a 50% fall in the firing rate produced by morphine (300 nM) or normorphine (300 nM) is really any indication of opiate tolerance in these cells? In tissue which has been incubated in normal Krebs solution, morphine (300 nM) reduced cell firing to less than 25% of the control level in 9 of 11 opiate sensitive cells. Therefore, failure of morphine to inhibit cell firing by at least 50% surely indicates development of tolerance. If the morphine was allowed to remain in the perfusing solution for periods exceeding 15 min, the inhibition was often seen to 'pass off' during the presence of the morphine. This was never observed with similar concentrations of morphine in control situations (Fig. 8). This finding may indicate that although the cells were sensitive to morphine as measured by short applications, there was evidence for a persisting underlying change.

The loss of sensitivity to naloxone during the first h after removal of morphine was apparently monotonic (Table 6). Within 60 min of switching the bathing solution from one which contained morphine to one which was free of drug, application of naloxone had only the same effect on the firing rate as on the control cells never exposed to morphine. The time course over which single myenteric neurons recover from opiate tolerance and lose the ability to be excited by naloxone closely follows the time course of analogous events demonstrated in the isolated guinea-pig ileum. Cox (1978b), while studying the nerve mediated contractile response, demonstrated two distinct phases in the recovery from opiate tolerance in the isolated guinea-pig ileum. One phase decayed rapidly after drug withdrawal while the second phase long outlasted the removal of the opiate from the bathing solution. Administration of naloxone in the first or rapidly decaying phase of toler-

ance induced a sustained contracture of the ileum but was unable to induce a contracture if administered during the second phase of tolerance (unless the tissue was again temporarily exposed to an opiate). The inference from this study was that within the myenteric plexus of the guinea-pig there is a homogenous population of opiate sensitive neurons and that each neuron exhibits two distinct phases in the recovery process.

The present investigation suggests another possible explanation. That is, the biphasic pattern in the recovery process observed by Cox may be due to the existence of two types of myenteric neurons. One type rapidly regained sensitivity (eg. Fig. 23) whilst the second type remained tolerant to morphine for a much longer time (eg. Fig. 22). Cox would have been unable to distinguish such neuronal types in his experiments on the ileum.

There are similarities in the time course of recovery from opiate tolerance in vivo and in isolated tissues. That is, the biphasic character of this process in myenteric neurons resembles the biphasic pattern characteristic of humans undergoing withdrawal (see section 1.1.3.). However, the duration of the phases of loss of tolerance in humans are measured in days and weeks rather than hours.

#### 4.3.4. In Vitro and In Vivo Exposures to Morphine --

##### A Comparison of Effects

The results from tissues which have been incubated in an opiate containing Krebs solution for 24-36 h did not differ noticeably from those

of tissues which had been removed from morphine pretreated guinea-pigs (and maintained in morphine containing Krebs solution). Morphine (2 - 30  $\mu\text{M}$ ) failed to inhibit the firing rate in 78% of the neurons exposed to morphine in vitro and 75% of the neurons exposed to morphine in vivo. Naloxone increased the firing rate in 79% of the neurons exposed to morphine in vitro and 70% of the neurons exposed to morphine in vivo. Thus, both methods of exposure are equivalent. The time course of recovery from opiate tolerance and 'dependence' induced by the two methods of exposure are also similar. A biphasic recovery pattern for opiate tolerance and a monophasic pattern for opiate 'dependence' was found with each method of exposure (Tables 5 and 6). The only apparent difference between the results from the tissues subjected to morphine in vitro or in vivo was the lower mean basal firing rate displayed by the tissues incubated in vitro. This finding has already been discussed (see section 4.3.1.). The present results are in agreement with those reported by Opmeer and van Ree (1978). These authors reported that opiate tolerance and 'dependence' induced in the isolated guinea-pig ileum in vitro was similar in many ways to that induced with in vivo exposure. In addition, these authors reported that the degree of opiate tolerance induced with in vitro exposure was dependent on the concentration of opiate in the incubation media (0.8 - 80  $\mu\text{M}$ ).

The mean plasma concentration of morphine in guinea-pigs implanted with morphine pellets was 4.7  $\mu\text{M}$  at the time of death. The concentration of morphine in the solution used to bath the myenteric plexus from these same animals was 200 - 500 nM. It is likely that perfusing the tissue with a concentration of morphine which is 10%

that of plasma levels may have affected the level of tolerance and the time course of recovery, although it has been reported that a dose of morphine in human subjects which is 20% that of the normal maintenance dose is sufficient to prevent any overt signs of opiate withdrawal (Martin, 1971). This concentration of morphine (200 - 500 nM) was used because it has been previously shown to correspond to the concentration of morphine in guinea-pig plasma at the time of tissue removal (Schulz and Herz, 1976a; Schulz and Goldstein, 1973). It is possible that the high value of the present determination was spurious due to inflammation at the site of implantation (of the pellets) which resulted in an increased rate of absorption, in turn yielding a higher plasma level.

Plasma levels of morphine from animals which were injected 3 times daily with morphine sulfate were considered artificially high because these samples of blood were taken only 2 h after the last injection. A more appropriate collection interval would have been 8 h post-injection.

#### 4.3.5. Opiate Tolerance and Dependence in other

##### In Vitro Systems

##### Neuroblastoma x glioma hybrid cells

Opiates inhibit adenylate cyclase activity in cultured neuroblastoma x glioma hybrid cells (NG108-15) (Sharma et al., 1975). When

these hybrid cells are cultured for many h in the presence of opiate the cells acquire tolerance to the drug. That is, cyclic-AMP levels which were originally depressed have now returned to normal values. Dependence on the opiate is demonstrated by the marked increase in cyclic-AMP levels produced by these cells when the opiate is rapidly withdrawn or naloxone is administered. The findings from cultured cells as well as those from whole animals studies (QMWS) (see section 1.1.4.) provide support for the involvement of the cyclic nucleotides in opiate tolerance and dependence. However, these studies suffer from certain difficulties with respect to their application; those concerning whole animal investigation already having been discussed (see section 1.1.4) Extrapolation of the results obtained from cultured hybrid cells to the level of the intact animal poses a certain difficulty; namely, how can results obtained from malignant tumor cells in vitro in any way apply to the intact, well differentiated and organized central nervous system of the adult mammal? This question has not been answered.

#### Cultured spinal cord-dorsal root ganglia

It was recently reported that opiates depressed the dorsal horn response in fetal mouse spinal cord-dorsal root ganglia (DRG) explants (Crain et al., 1977). When such cord-DRG cultures were incubated (at 35°C) in morphine (1  $\mu$ M) for 2-3 days the acute depressant action of morphine on sensory evoked dorsal horn responses gradually disappeared and increasing the concentration of morphine to 100  $\mu$ M was without effect. Thus, tolerance to the acute effect of morphine was demonstrated (as was cross-tolerance to met-enkephalin) (Crain, Crain, Finnigan and Simon, 1979). Development of dependence was also suggested

by the enhanced dorsal horn responses in tolerant cultures. The tolerance induced with in vitro exposure was long lasting (several days) after removal of the morphine but could not be induced with incubations at 20°C.

These findings from cord-DRG explants confirm the present study on the myenteric plexus; that is, in vitro development of opiate tolerance and dependence in nervous tissue. However, the temperature-dependence of the opiate tolerance mechanism (which suggests metabolic requirements) and the long duration of tolerance seen in the cord-DRG explants is in contrast to the opiate tolerance which develops in myenteric neurons (present study). Such differences may be due in part to differences in the processes involved in the development of opiate tolerance in central vs. peripheral neurons as well as differences in the stage of maturity of the neurons (differentiated vs. undifferentiated).

Indeed, one significant advantage the myenteric plexus has over the fetal spinal cord-DRG explant or neuroblastoma x glioma hybrid cells as a model to study the acute and chronic action of opiates is its full differentiation. With respect to this concept, results obtained from myenteric neurons may more closely resemble the actual opiate response in the adult CNS.



#### 4.3.6. Alteration of the Development of Opiate Tolerance and

##### 'Dependence' -- Concomitant Incubations with other Drugs

Segments of ileum were incubated concomitantly with morphine in addition to agents believed to affect either protein synthesis, synaptic transmission or cyclic nucleotide metabolism. The purpose of these experiments was to determine whether the induction of opiate tolerance and 'dependence' in single neurons was somehow dependent on one or more of these factors.

Protein synthesis inhibitors have been previously shown to suppress the development of opiate tolerance and dependence in whole animals and to partially suppress this development in isolated tissues (Sharma et al., 1975; Hammond et al., 1976). In the present study it was found that incubation of ileum in solutions containing cycloheximide and morphine did not prevent the development of opiate tolerance. That is, there was no difference in opiate sensitivity between tissue incubated in solutions containing morphine alone and morphine plus cycloheximide. However, the response to naloxone was affected by the presence of cycloheximide in the incubation medium. Fewer neurons were excited by naloxone and this excitation was of a smaller magnitude when tissue was incubated with both morphine and cycloheximide as opposed to morphine alone. Two explanations for these findings are offered. First, in the isolated guinea-pig ileum, protein synthesis inhibitors (i.e. cycloheximide) affect the development of opiate 'dependence' without affecting the development of opiate tolerance. Second, protein synthesis inhibitors exert their modulatory effect by inhibiting the expression

of dependence (neuronal excitation) and not by interfering with the underlying mechanism. This action may be secondary due to uncoupling of oxidative phosphorylation (Robbins, 1974), fall in intracellular pH (Robbins, 1974) or changes in the synthesis of opiate receptors. On the other hand, it seems likely that the development of opiate tolerance and 'dependence' in single myenteric neurons (as distinct from the expression) does not require protein synthesis. Evidence for this was the finding that opiate tolerance and 'dependence' could be induced in isolated ileum at 40°C (Hammond et al., 1976), a temperature at which a minimal amount of protein synthesis might be expected to be taking place (see section 1.4.5.).

Agents affecting synaptic transmission were similarly tested as to their ability to modify the induction of opiate tolerance and 'dependence'. Tissues were incubated in solutions containing morphine and lidocaine; or morphine, hexamethonium and hyoscine. Lidocaine prevented all neuronal activity throughout the 24 h period (see section 3.7.1.2.): and hexamethonium and hyoscine block completely cholinergic synaptic transmission in the myenteric plexus (see section 3.7.1.1.). The results obtained from tissues subjected to these conditions did not differ from tissues incubated in morphine alone. The present results therefore indicate that long term changes induced by morphine in single myenteric neurons are not dependent on ongoing neuronal activity or synaptic transmission.

The present finding does not support those theories suggesting changes in synaptic transmission as the underlying mechanism of opiate tolerance and dependence. Collier (1968) advanced the idea that super-

sensitivity of the postsynaptic cell to the presynaptic transmitter could be induced by morphine if it acted by suppressing synaptic transmission. This drug-induced supersensitivity could account both for aspects of opiate tolerance and abstinence. Release of neurotransmitter would remain depressed throughout the exposure to the morphine but changes in the postsynaptic cell would compensate for this effect much like the changes observed with denervation supersensitivity (Thesleff, 1960); as a result, tolerance to morphine develops, because, even in the presence of morphine, a normal response is observed. If, on withdrawing the morphine, normal release of transmitter is restored, then a response which opposes that seen when opiates are given acutely should be observed (abstinence). However, in the isolated myenteric plexus there is an absence of any ongoing synaptic activity (Nishi and North, 1973a), and it is well established that the action of opiates is directly on the cell under investigation (Dingledine et al., 1976). The present study indicates that opiate tolerance and dependence can be induced at the cellular level without ongoing neuronal activity or synaptic transmission, and therefore, argues against the theory of Collier (1968).

When tissues were incubated in solutions containing morphine, dbc-AMP and IBMX the results obtained were inconclusive. There was no difference between sensitivity of neurons in such tissue to opiates and that of neurons incubated in morphine alone; however, less than 50% of the neurons tested with naloxone responded with an excitation and the degree of excitation was small. This contrasts with the marked excitations produced by naloxone in tissue incubated in solutions lacking agents which may affect cyclic nucleotide levels. At least two possible explanations for this finding exist. First, it is possible that in-

creasing intracellular levels of cyclic nucleotides (cyclic-AMP) suppresses the development of opiate 'dependence' without affecting the development of opiate tolerance. Second, altering cyclic nucleotide levels may affect the development of neither. The acute action of dbc-AMP + IBMX on myenteric neurons was inhibition of cell firing. Indeed, the mean basal firing rate of neurons incubated in dbc-AMP + IBMX was lower than that of control incubated tissue (see section 3.7.3.1.). Perhaps the manifestation of 'dependence' (naloxone induced excitation) was attenuated because of an ongoing depression of neuronal activity caused by these compounds. In other words, IBMX and dbc-AMP have no effect on the underlying mechanism of opiate dependence but exert their modulatory effects through a physiological antagonism: IBMX and dbc-AMP to inhibit these cells and naloxone to excite them.

In any event, further investigation on the effects of the cyclic nucleotides in the mechanism underlying opiate tolerance and dependence should be performed prior to the assessment of a role. Such an investigation should look at the time course of events leading to and the recovery from this state.

#### 4.3.7. Cellular Mechanism of Opiate 'Dependence'

##### Speculation -- Enhanced Neuronal Excitability

It has been suggested that the morphine withdrawal syndrome reflects a state of rebound hyperexcitability of the CNS (Jaffe and Sharp-

less, 1968). This withdrawal syndrome should not be considered a unitary phenomenon but as an ensemble of signs which probably reflect the intense activation of a number of brain sites. The neurotransmitter systems which have been implicated in opiate withdrawal are the cholinergic (Frederickson, 1975), dopaminergic (Lal and Numan, 1976) and noradrenergic (Herz et al., 1974) systems. Atropine, haloperidol and propranolol have all been shown to alter the severity of the withdrawal syndrome. Consistent with this idea of neuronal hyperexcitability is an increase in activity of myenteric neurons (which have been chronically exposed to morphine) by simply withdrawing the morphine from the bathing solution or by administering naloxone. The underlying mechanism for this increase in firing is most likely a depolarization of the cell membrane (Johnson and North, 1980).

The therapeutic implications of this knowledge of hyperexcitability for the management of opiate withdrawal are far reaching especially if the directly affected neurons can be identified. Drugs acting to stabilize the neuronal membrane or to produce CNS depression would most likely be effective agents in alleviating discomforting signs of abstinence. Indeed, clonidine, a central noradrenergic agonist with potent inhibitory action in the brain (Svensson, Bunney and Aghajanian, 1975) and guinea-pig ileum (Werner, Starke and Schuman, 1972) have been used to successfully withdraw subjects from a state of chronic opiate addiction (Gold, Redmond and Kleber, 1978). Clonidine has also been shown to suppress signs of morphine withdrawal in single neurons of the rat locus coeruleus (Aghajanian, 1978). Agents which act by stabilizing the cell membrane, for example the tricyclic antidepressants, are

also very effective in suppressing signs of morphine withdrawal in mice (Contreras, Tamayo and Quijada, 1977).

Clinical manifestations of the withdrawal reaction are in a direction which opposes that seen when morphine is given acutely to drug naive individuals. This finding suggests that Himmelsbach's homeostatic counteradaptive theory is still operative. Whatever the underlying mechanism, the ultimate withdrawal reaction seems to be the result of an increased excitability of structure and pathways mediating specific physiological effects.

#### 4.3.8. General Conclusions

Tolerance and 'dependence' can be induced in single mammalian neurons exposed to morphine in vitro. This action was mediated through the opiate receptor since levorphanol but not dextrorphan induced the response. The time course of recovery from opiate tolerance and 'dependence' was studied, and their pattern of recovery was shown to be multiphasic - similar to the pattern observed in human addicts. Two types of myenteric neurons were observed with respect to recovery from opiate tolerance. The present findings support the validity of the MPLM preparation as a useful model to study the underlying changes associated with opiate tolerance and dependence. The inability of protein synthesis inhibitors or agents affecting synaptic transmission to modify the development of opiate tolerance and dependence argues against their

involvement in the underlying mechanism. The results with agents affecting cyclic nucleotide metabolism were less clear. The results obtained with in vivo exposure to morphine were not different from the results obtained with in vitro incubations.

The present experiments have helped in understanding the basic change in the properties of neurons in the tolerant/dependent state. The fact that naloxone so greatly excited these cells (whereas it normally is without effect) implies that the primary change underlying the withdrawal state is rebound neuronal excitability. There is recent evidence suggesting that the mechanism underlying this enhanced excitability is a depolarization of the cell membrane. It is likely that drugs affecting membrane properties (eg. membrane stabilizing drugs) or drugs which selectively depress the activity of opiate sensitive cells (eg. clonidine) will provide a pharmacological means to detoxify and stabilize drug addicts without themselves producing dependence. The therapeutic implications of such drugs are far reaching in light of the fact that over 500,000 Americans are currently dependent on narcotics.

#### 4.4. EXTRACELLULAR RECORDINGS OF MYENTERIC NEURONS -- LIMITATIONS

Extracellular recordings afford one the opportunity to measure directly the activity of single neurons and the action of drugs which may affect this activity (see section 1.). When applied to the myenteric plexus longitudinal muscle preparation this technique suffers from 3 major criticisms. One, the firing rate of neurons was often variable and sporadic; this does not allow for easy interpretation of drug effects which may be slight. That is, it was difficult to ascribe a small effect to a drug because of the variability of neuronal firing. Two, successful recordings of single myenteric neurons usually lasted only 1-2 h so drug effects which were slow in onset and offset were not easily measured; for example, the action of buprenorphine on single myenteric neurons (see above). Three, extracellular recording techniques do not easily lend themselves to quantification of drug effects. This point should be stressed since it is an important one. The large variability in opiate sensitivity and neuronal firing rate amongst the myenteric neurons makes statistical comparisons of limited value unless very large samples are used; on the other hand, effects of drugs on a given neuron are clear, dose-dependent and readily reproducible when recording conditions permit.



#### 4.5. PROPOSALS FOR FUTURE RESEARCH

The present experiments represent a preliminary approach in the use of this technique to study the long term effects of opiates. Three proposals for future studies are offered. First, a careful study of those drugs which may modify the development or expression of opiate dependence in the whole animal should be performed at the level of the single mammalian neuron. In this way it will be possible to determine whether these drugs are effective directly at the cellular level or by some indirect mechanism. Particular attention should be given to those drugs which are thought to stabilize the cell membrane. Second, an in vivo study is required of the long term effects of opiates on several brain sites. The action of opiate agonists and antagonists at these sites should be studied to determine whether these sites demonstrate characteristics of both opiate tolerance and 'dependence'; or, as suggested by the lesion studies, aspects of only tolerance or of only dependence. Third, intracellular recordings of neurons should be performed so as to help elucidate the mechanisms underlying the changes brought on by such long term exposures to opiates.

## REFERENCES

- Ambache, N. (1954). Separation of the longitudinal muscle of the rabbit ileum as a broad sheet. *J. Physiol.*, 125, 53-55.
- Aghajanian, G.K. (1978). Tolerance of locus coeruleus neurones to morphine and suppression of withdrawal responses by clonidine. *Nature*, 276, 186-188.
- Anderson, S.D., Basbaum, A.I. & Fields, H.L. (1977). Response of medullary raphe neurons to peripheral stimulation and to systemic opiates. *Brain Res.*, 123, 363-368.
- Atweh, S.F., Murrin, L.C. & Kuhar, M.J. (1978). Presynaptic localization of opiate receptors in the vagal and accessory optic systems: an autoradiographic study. *Neuropharmac.*, 17, 65-71.
- Axelrod, J. (1956). Possible mechanisms of tolerance to narcotic drugs. *Science*, 124, 263-264.
- Basbaum, A.I. & Fields, H. (1978). Endogenous pain control mechanisms: review and hypothesis. *Ann Neurol.*, 4, 451-462.
- Beavo, J.A., Rogers, N.L., Crofford, O.B., Hardman, J.G., Sutherland, E.W. & Newman, E.V. (1970). Effects of xanthine derivatives on lipolysis and on adenosine-3', 5'-monophosphate diesterase activity. *Mol. Pharmac.*, 6, 597-603.
- Berkowitz, B. & Spector, S. (1972). Evidence for active immunity to morphine in mice. *Science*, 178, 1290-1292.
- Bird, S.J. & Kuhar, M.J. (1977). Iontophoretic application of opiates to the locus coeruleus. *Brain Res.*, 122, 523-533.
- Bonnet, K.A. (1975). Regional alterations in cyclic nucleotide levels with acute and chronic morphine treatment. *Life Sci.*, 16, 1877-1882.
- Bonnet, K.A., Hiller, J.M. & Simon, E.J. (1976). The effects of chronic opiate treatment and social isolation of opiate receptors in rodent brain. In Opiates and Endogenous Opioid Peptides. ed. Kosterlitz, H.W. pp. 335-343. Amsterdam: Elsevier.
- Bradley, P.B., Gayton, R.J. & Lambert, L.A. (1978). Electrophysiological effects of opiates and opioid peptides. In Centrally Acting Peptides. ed. Hughes, J. pp. 215-229. Baltimore: University Park.

- Calvillo, O., Henry, J.L. & Neuman, R.S. (1974). Effects of morphine and naloxone on dorsal horn neurones in the cat. *Can. J. Physiol., Pharmac.*, 54, 1207-1211.
- Chang, K.J., Cooper, B.R., Hazum, E. and Cuatrecasas, P. (1979). Multiple Opiate Receptors: different regional distribution in the brain and differential binding of opiates and opioid peptides. *Mol. Pharmac.*, 16, 91-104.
- Childers, S.R., Simantov, R. & Snyder, S.H. (1977). Enkephalin: radio-immunoassay and radioreceptor assay in morphine dependent rats. *Europ. J. Pharmac.*, 46, 289-293.
- Cochin, J. (1970). Possible mechanisms in development of tolerance. *Fedn. Proc.*, 29, 19-29.
- Cochin, J., Haggart, J., Woods, L.A. & Seevers, M.H. (1954). Plasma levels, urinary and fecal excretion of morphine in non-tolerant and tolerant dogs. *J. Pharmac. exp. Ther.*, 111, 74-83.
- Cochin, J. & Kornetsky, C. (1964). Development and loss of tolerance to morphine in the rat after single and multiple injections. *J. Pharmac. exp. Ther.*, 145, 1-10.
- Collier, H.O.J. (1968). Supersensitivity and dependence. *Nature*, 220, 228-231.
- Collier, H.O.J. (1980). Cellular site of opiate dependence. *Nature*, 283, 625-629.
- Collier, H.O.J. & Francis, D.L. (1975). Morphine abstinence tolerance and dependence is associated with increased brain cyclic AMP. *Nature*, 255, 159-162.
- Collier, H.O.J., Francis, D.L., Henderson, G. & Schneider, C. (1974). Quasi morphine-abstinence syndrome. *Nature*, 249, 471-473.
- Collier, H.O.J. & Roy, A.C. (1974). Morphine-like drugs inhibit the stimulation by E prostaglandins of cyclic AMP formation by rat brain homogenate. *Nature*, 248, 24-27.
- Contreras, E., Tamayo, L. and Quizada, L. (1977). Effects of tricyclic compounds and other drugs having a membrane stabilizing action on analgesia, tolerance to and dependence on morphine. *Arch. Int. Pharmacodyn. Ther.*, 228, 293-299.
- Cook, R. & Burnstock, G. (1976). The ultrastructure of Auerbach's plexus in the guinea pig. I. neuronal elements. *J. Neurocytol.*, 5, 171-194.
- Costa, M., Patel, Y., Furness, J.B. & Arimura, A. (1977). Evidence that some intrinsic neurons of the intestine contain somatostatin. *Neurosci. Letters*, 6, 215-222.

- Cowan, A., Lewis, J.W. & Macfarlane, I.R. (1977). Agonist and antagonist properties of buprenorphine, a new antinociceptive agent. *Br. J. Pharmac.*, 60, 537-545.
- Cox, B.M. (1978a). Stimulus-response relationships in ileum preparations from normal and morphine treated guinea pigs. *Br. J. Pharmac.*, 62, 387-388P.
- Cox, B.M. (1978b). Multiple mechanisms in opiate tolerance. In *Characteristics and Functions of Opioids*. eds. van Ree, J.M. and Terenius, L. pp. 13-24. Amsterdam: Elsevier.
- Cox, B.M. & Osman, O.H. (1970). Inhibition of the development of tolerance to morphine in rats by drugs which inhibit ribonucleic acid or protein synthesis. *Br. J. Pharmac.*, 38, 157-170.
- Cox, B.M. & Padhya, R. (1977). Opiate binding and effect in ileum preparations from normal and morphine pretreated guinea pigs. *Br. J. Pharmac.*, 61, 271-278.
- Crain, S.M., Crain, B., Finnigan, T. & Simon, E.J. (1979). Development of tolerance to opiates and opioid peptides in organotypic cultures of mouse spinal cord. *Life Sci.*, 25, 1797-1802.
- Crain, S.M., Peterson, E.R., Crain, B. & Simon, E. (1977). Selective opiate depression of sensory-evoked synaptic networks in dorsal horn regions of spinal cord cultures. *Brain Res.*, 133, 162-166.
- Creese, I. & Snyder, S.H. (1975). Receptor binding and pharmacological activity of opiates in the guinea-pig intestine. *J. Pharmac. exp. Ther.*, 194, 205-219.
- Dafny, N., Brown, M., Burks, T.F. & Rigor, B.M. (1979). Unit activity recorded simultaneously from medial thalamus and caudate nucleus in naive and morphine-dependent rats. *Exp. Neur.*, 64, 216-224.
- Davies, J. (1976). Effects of morphine and naloxone on Renshaw cells and spinal interneurons in morphine dependent and nondependent rats. *Brain Res.*, 113, 311-326.
- Davies, J. and Dray, A. (1978). Pharmacological and electrophysiological studies of morphine and enkephalin on rat supraspinal neurones and cat spinal neurones. *Br. J. Pharmac.*, 63, 87-96.
- Davies, M.E., Akera, T. and Brody, T.M. (1975). Saturable binding of morphine to rat brain-stem slices and the effect of chronic morphine treatment. *Res. Commun. Chem. Path. Pharmac.*, 12, 409-418.
- Dingledine, R. & Goldstein, A. (1975). Single neuron studies of opiate action in the guinea-pig myenteric plexus. *Life Sci.*, 17, 57-62.

- Dingledine, R. & Goldstein, A. (1976). Effect of synaptic transmission blockade on morphine action in the guinea-pig myenteric plexus. *J. Pharmac. exp. Ther.*, 196, 97-106.
- Dingledine, R., Goldstein, A. & Kendig, J. (1974). Effects of narcotic opiates and serotonin on the electrical behavior of neurons in the guinea-pig myenteric plexus. *Life Sci.*, 14, 2299-2309.
- Dingledine, R., Iverson, L.L. & Breuker, E. (1978). Naloxone as a GABA antagonist: evidence from iontophoretic, receptor binding and convulsant studies. *Europ. J. Pharmac.*, 47, 19-27.
- Dole, V.P. & Nyswander, M. (1965). A medical treatment for diacetylmorphine (heroin) addiction. *J. Amer. Med. Assoc.*, 193, 646-650.
- Dreyfus, C.F., Bornstein, M.B. & Gershon, M.D. (1977). Synthesis of serotonin by neurons of the myenteric plexus in situ and in organotypic tissue culture. *Brain Res.*, 128, 125-139.
- Dreyfus, C.F., Sherman, D.L. & Gershon, M.D. (1977). Uptake of serotonin by intrinsic neurons of the myenteric plexus grown in organotypic tissue culture. *Brain Res.*, 128, 109-123.
- Duggan, A.W. & Grierson, B.T. (1979). Methyl xanthine, adenosine 3',5'-cyclic monophosphate and the spinal transmission of nociceptive information. *Br. J. Pharmac.*, 67, 51-57.
- Duggan, A., Hall, J. & Headley, P. (1976). Morphine, enkephalin and the substantia gelatinosa. *Nature*, 264, 456-458.
- Duggan, A.W., Hall, T. & Headley, P. (1977a). Enkephalins and dorsal horn neurones of the cat: effects on responses to noxious and innocuous skin stimuli. *Br. J. Pharmac.*, 61, 399-408.
- Duggan, A.W., Hall, J.G. & Headley, P.M. (1977b). Suppression of transmission of nociceptive impulses by morphine: selective effects of morphine administered in the region of the substantia gelatinosa. *Br. J. Pharmac.*, 61, 65-76.
- Ehrenpreis, S., Greenberg, J. & Belman, S. (1973). Prostaglandins reverse inhibition of electrically induced contractions of guinea-pig ileum by morphine, indomethacin and acetylsalicylic acid. *Nature*, 245, 280-282.
- Ehrlich, P. (1960). On partial functions of the cell. In Collected Papers of Paul Ehrlich. Vol. 3. London: Pergamon.
- Eidelberg, E. & Bond, M.L. (1972). Effects of morphine and antagonists on hypothalamic cells activity. *Archs. Int. Pharmacodyn. Ther.*, 196, 16-24.

- Elde, R., Hokfelt, T., Johansson, O. & Terenius, L. (1976). Immuno-histochemical studies using antibodies to leucine-enkephalin: initial observations on the nervous system of the rat. *Neurosci.*, 1, 349-351.
- Erwin, D., Nonchoji, T. & Wood, J. (1978). Effects of morphine on electrical activity of single myenteric neurons in cat small bowel. *Europ. J. Pharmac.*, 47, 401-405.
- Feinberg, M.P. & Cochin, J. (1977). Studies on tolerance. II. The effect of timing on inhibition of tolerance to morphine by cycloheximide. *J. Pharmac. exp. Ther.*, 203, 332-339.
- Feldberg, W. & Lin, R.C.Y. (1950). Synthesis of acetylcholine in the wall of the digestive tract. *J. Physiol.*, 111, 96-118.
- Ferri, S., Santagostino, A., Braga, P.C. & Galatulas, I. (1974). Decreased antinociceptive effect of morphine in rats treated intraventricularly with prostaglandin E<sub>1</sub>. *Psychopharmacol.*, 39, 231-235.
- Fields, H.F., Basbaum, A.I., Clanton, C.H. & Anderson, S.D. (1977). Nucleus raphe magnus inhibition of dorsal horn neurons. *Brain Res.*, 126, 441-453.
- Fleming, W.W. (1976). Variable sensitivity of excitable cells: possible mechanisms and biological significance. In Reviews of Neuroscience. eds. Ehrenpreis, S. and Kopin, I.J. pp. 43-90. New York: Raven Press.
- Francis, D.L., Cuthbert, N.J., Butt, N.M. & Collier, H.O.J. (1978). Meaning of quasi-withdrawal phenomena for the cellular mechanism of abstinence. In Characteristics and Function of Opioids. eds. van Ree, J. and Terenius, L. pp. 37-49. Amsterdam: Elsevier.
- Francis, D.L., Cuthbert, N.J., Saeed, S.A., Butt, N.M. & Collier, H.O.J. (1978). Meaning of quasi-withdrawal phenomena for the cellular mechanism of abstinence. In Characteristics and Functions of Opioids. eds. van Ree, J.M. and Terenius, L. pp. 37-50. Amsterdam: Elsevier.
- Francis, D.L., Roy, A.C. & Collier, H.O.J. (1975). Morphine abstinence and quasi-abstinence effects after phosphodiesterase inhibitors and naloxone. *Life Sci.*, 16, 1901-1906.
- Fraser, H.F. & Isbell, H. (1952). Comparative effects of 20 mg of morphine sulfate on non-addicts and former morphine addicts. *J. Pharmac. exp. Ther.*, 105, 498-502.
- Fraser, H.F. & Isbell, H. (1960). Pharmacology and addiction liability of dl- and d-propoxyphene. *Bull. Narcot.*, 12, 9-14.

- Fraser, H.F. & Rosenberg, D.E. (1966). Comparative effects of (I) chronic administration of cyclazocine, (II) substitution of nalorphine for cyclazocine and, (III) chronic administration of morphine. Pilot crossover study. *Int. J. Addict.*, 1, 86-98.
- Fratta, W., Yang, H.-Y.T., Hong, J. & Costa, E. (1977). Stability of met-enkephalin content in brain structures of morphine-dependent or foot-shock stressed rats. *Nature*, 268, 452-453.
- Frederickson, R.C.A. (1975). Morphine withdrawal response and central cholinergic activity. *Nature*, 257, 131-132.
- Frederickson, R., Hewes, C. & Aken, J.W. (1976). Correlation between the in vivo and in vitro expression of opiate withdrawal precipitated by naloxone: Their antagonism by  $\Delta^9$ -tetrahydrocannabinol. *J. Pharmac. exp. Ther.*, 199, 375-384.
- Frederickson, R.C.A., Horng, J.C., Burgis, V. & Wong, D.T. (1974). Alteration of opiate receptors in physically dependent rats. In Problems of Drug Dependence. pp. 411-434. Washington: National Academy of Sciences.
- Frederickson, R.C.A., Norris, F.H. & Hewes, C. (1975). Effects of naloxone and acetylcholine on medial thalamic and cortical units in naive and morphine dependent rats. *Life Sci.*, 17, 81-82.
- Fry, J.P., Zieglgänsberger, W. & Herz, A. (1978). Tachyphylaxis to enkephalin - tolerance and dependence at the single neurone level. In Iontophoresis and Transmitter Mechanisms in the Mammalian Central Nervous System. eds. Ryall, R.W. and Kelly, J.S. pp. 323-325. Amsterdam: Elsevier.
- Furness, J.B. (1969). The presence of inhibitory nerves in the colon after sympathetic denervation. *Europ. J. Pharmac.*, 6, 349-353.
- Gabella, G. (1972). Fine structure of the myenteric plexus in the guinea pig ileum. *J. Anat.*, 111, 69-97.
- Gabella, G. (1976). Structure of The Autonomic Nervous System. New York: Halsted Press.
- Gilbert, P.E. and Martin, W.R. (1976). The effects of morphine- and nalorphine-like drugs in the nondependent, morphine-dependent and cyclazocine-dependent chronic spinal dog. *J. Pharmac. exp. Ther.*, 198, 66-82.
- Gintzler, A.R. & Musacchio, J.M. (1975). Interactions of morphine, adenosine, adenosine triphosphate and phosphodiesterase inhibitors on the field stimulated guinea-pig ileum. *J. Pharmac. exp. Ther.*, 194, 575-582.

- Glick, S.D. & Charap, A.D. (1973). Morphine dependence in rats with medial forebrain bundle lesions. *Psychopharmacology*, 30, 343-348.
- Glick, S.D. & Crane, L.A. (1978). Opiate-like and abstinence-like effects of intracerebral histamine administration in rats. *Nature*, 273, 547-549.
- Gold, M.S., Redmond, D.E. & Kleber, H.D. (1978). Clonidine in opiate withdrawal. *Lancet*, 1, 929.
- Goldstein, A. & Sheehan, P. (1969). Tolerance to opioid narcotics. I. Tolerance to the "running jet" caused by levorphanol in the mouse. *J. Pharmac. exp. Ther.*, 169, 175-184.
- Greenberg, R., Kosterlitz, H.W. & Waterfield, A.A. (1970). The effects of hexamethonium, morphine and adrenaline on the output of acetylcholine from the myenteric plexus-longitudinal muscle preparation of the ileum. *Br. J. Pharmac.*, 40, 553-554P.
- Gutfreund, H. (1965). An Introduction to the Study of Enzymes. Great Britain: Scientific Publications.
- Gyang, E.A. & Kosterlitz, H.W. (1966). Agonist and antagonist actions of morphine-like drugs on the guinea pig isolated ileum. *Br. J. Pharmac. Chemother.*, 27, 514-527.
- Haigler, H.J. (1976). Morphine: ability to block neuronal activity evoked by a nociceptive stimulus. *Life Sci.*, 19, 841-858.
- Hambrook, J.M. & Rance, M.J. (1976). The interaction of buprenorphine with the opiate receptor: lipophilicity as a determining factor in drug-receptor kinetics. In Opiates and Endogenous Opioid Peptides. ed. Kosterlitz, H.W. pp. 295-301. Amsterdam: Elsevier.
- Hammond, M.D., Schneider, C. & Collier, H.O.J. (1976). Induction of opiate tolerance in isolated guinea-pig ileum and its modification by drugs. In Opiates and Endogenous Opioid Peptides. ed. Kosterlitz, H.W. pp. 169-176. Amsterdam: Elsevier.
- Harris, R.A., Bhargava, H.N., Loh, H.H. & Way, E.L. (1974). Alteration of narcotic effects by calcium and other ions. *Fedn. Proc.*, 33, 515.
- Hayashi, E., Mori, M., Yamada, W. & Kunitomo, M. (1978). Effects of purine compounds on cholinergic nerves. Specificity of adenosine and related compounds on acetylcholine release in electrically stimulated guinea-pig ileum. *Eur. J. Pharmac.*, 48, 297-307.
- Henderson, G. & Hughes, J. (1976). The effects of morphine on the release of noradrenaline from the mouse vas deferens. *Br. J. Pharmac.*, 57, 551-557.



- Henderson, G. & North, R.A. (1975). Presynaptic action of 5-hydroxytryptamine in the myenteric plexus of the guinea-pig ileum. *Br. J. Pharmac.*, 54, 265P.
- Herndon, B.L., Baeder, D.H. & Ringle, D.A. (1974). An immunoglobulin model of the morphine receptor. *Fedn. Proc.*, 33, 571.
- Herz, A., Albus, K., Metys, T., Schubert, P. & Teschemacher, H. (1970). On the central sites for the antinociceptive action of morphine and fentanyl. *Neuropharmac.*, 9, 539-551.
- Herz, A., Bläzigg, J. & Papeschi, R. (1974). Role of catecholaminergic mechanisms in the expression of the morphine abstinence syndrome in rats. *Psychopharmac.*, 39, 121-143.
- Herz, A., Teschemacher, H., Albus, K. & Zieglgänsberger, S. (1972). Morphine abstinence syndrome in rabbits precipitated by injection of morphine antagonists into the ventricular system and restricted parts of it. *Psychopharmac.*, 26, 219-235.
- Hill, R.G. & Pepper, C.M. (1978). Selective effects of morphine on the nociceptive responses of thalamic neurones in the rat. *Br. J. Pharmac.*, 64, 137-143.
- Himmelsbach, C.K. (1943). Morphine, with reference to physical dependence. *Fedn. Proc.*, 2, 201-203.
- Hirst, G.D., Holman, M.E. & Spence, I. (1974). Two types of neurons in the myenteric plexus of duodenum in the guinea-pig. *J. Physiol.*, 236, 303-326.
- Hirst, G.D.S. & McKirdy, H.D. (1974). Presynaptic inhibition at mammalian peripheral synapse? *Nature*, 250, 430-431.
- Ho, I.K., Loh, H.H. & Way, E.L. (1973a). Cyclic adenosine monophosphate antagonism of morphine analgesia. *J. Pharmac. exp. Ther.*, 185, 336-337.
- Ho, I.K., Loh, H.H. & Way, E.L. (1973b). Effects of cyclic 3',5'-adenosine monophosphate on morphine tolerance and physical dependence. *J. Pharmac. exp. Ther.*, 185, 347-357.
- Hug, C.C. (1972). Characteristics and theories related to acute and chronic tolerance development. In Chemical and Biological Aspects of Drug Dependence. pp. 307-358. Ohio: CRC Press.
- Hughes, J., Kosterlitz, H.W., Robson, E. & Waterfield, A.A. (1978). The inhibitory effects of clonidine on the contractions of the guinea pig ileum in the morphine-dependent and withdrawn states. *Br. J. Pharmac.*, 62, 388P.

- Hutchinson, M., Kosterlitz, H.W. & Leslie, F.M. (1975). Assessment in the guinea-pig ileum and mouse vas deferens of benzomorphans which have strong antinociceptive activity but do not substitute for morphine in the dependent monkey. *Br. J. Pharmac.*, 55, 541-546.
- Isbell, H. & Chrusciel, T.L. (1970). Dependence liability of "non-narcotic" drugs. *Bull. Wld. Hlth. Org.*, 43, (suppl.) 1-111.
- Jacobowitz, D. (1965). Histochemical studies of the autonomic innervation of the gut. *J. Pharmac. exp. Ther.*, 149, 358-364.
- Jaffe, J.H. (1968). Drug addiction and drug abuse. In *The Pharmacological Basis of Therapeutics*. eds. Goodman, L.S. and Gilman, A. pp. 285-311. New York: Macmillan.
- Jaffe, J.H. (1975). Drug addiction and drug abuse. In *The Pharmacological Basis of Therapeutics*. eds. Goodman, L.S. and Gilman, A. pp. 284-324. New York: Macmillan.
- Jaffe, J.H. & Martin, W.R. (1975). Narcotic analgesics and antagonists. In *The Pharmacological Basis of Therapeutics*. eds. Goodman, L.S. and Gilman, A. pp. 245-283. New York: Macmillan.
- Jaffe, J. & Sharpless, S. (1968). Pharmacological denervation supersensitivity in the central nervous system: a theory of physical dependence. *Proc., Ass., Res., ner., ment. Dis.*, 46, 226-246.
- Jasinski, D.R., Martin, W.R. & Hoeldtke, R.D. (1970). Effects of short- and long-term administration of pentazocine in man. *Clin. Pharmac. Ther.*, 11, 385-403.
- Jasinski, D.R., Martin, W.R. & Hoeldtke, R. (1971). Studies of the dependence-producing properties of GPA-1657, profadol and propiram in man. *Clin. Pharmac. Ther.*, 12, 613-649.
- Jasinski, D.R., Martin, W.R. & Sapira, J.D. (1968). Antagonism of the subjective, behavioral, pupillary and respiratory depressant effects of cyclazocine by naloxone. *Clin. Pharmac. Ther.*, 9, 215-222.
- Jasinski, D.R., Pevnick, J.S. & Griffith, J.D. (1978). Human pharmacology and abuse potential of the analgesic buprenorphine. *Arch. Gen. Psych.*, 35, 501-516.
- Johnson, S.M. & North, R.A. (1980). Membrane potential changes in neurones undergoing withdrawal from opiates. *Brain Res.* (in press).
- Johnson, S.M., Westfall, D.P., Howard, S.A. & Fleming, W.W. (1978). Sensitivities of the isolated ileal longitudinal smooth muscle - myenteric plexus and hypogastric nerve vas-deferens of the guinea pig after chronic morphine pellet implantation. *J. Pharmac. exp. Ther.*, 204, 54-66.

- Kakunaga, T., Kaneto, H. & Hano, K. (1966). Pharmacologic studies on analgesics. VII. Significance of the calcium ion in morphine analgesia. *J. Pharmac. exp. Ther.*, 153, 134-141.
- Kaneto, H. (1971). Inorganic ions: The role of calcium. In *Narcotic Drugs*. ed. Clouet, D.H. pp. 300-309. New York: Plenum Press.
- Katayama, Y. and North, R.A. (1978). Does substance P mediate slow synaptic excitation within the myenteric plexus? *Nature*, 274, 387-388.
- Katz, J.B. & Catravas, G.N. (1977). Absence of morphine antagonism of prostaglandin  $E_1$  - stimulated  $\{^3H\}$  3',5'-cyclic adenosine monophosphate accumulation in a rat brain mince system. *Brain Res.*, 120, 263-268.
- Kaymakcalan, S., Hakkiayhan, I. & Cankattulany, F. (1977). Naloxone-induced or postwithdrawal abstinence signs in  $\Delta^9$ -tetrahydrocannabinol-tolerant rats. *Psychopharmac.*, 55, 243-249.
- Kerr, F.W.L. & Pozuelo, J. (1971). Suppression of physical dependence and induction of hypersensitivity to morphine by stereotaxic hypothalamic lesions in addicted rats. *Proc. Mayo Clin.*, 46, 653-665.
- Kitahata, L.M., Kosaka, Y., Taub, A., Bonikos, K. & Hoffert, M. (1974). Lamina-specific suppression of dorsal-horn unit activity by morphine sulphate. *Anesth.*, 41, 39-48.
- Klee, W.A. (1978). Dual regulation of adenylate cyclase: a biochemical model for opiate tolerance and dependence. In *The Bases of Addiction*. ed. Fishman, J. pp. 431-440. Berlin: Dahlem Konferenzen.
- Klee, W.A., Sharma, S.K. & Nirenberg, M. (1975). Opiate receptors as regulators of adenylate cyclase. *Life Sci.*, 16, 1869-1874.
- Klee, W.A. & Streaty, R.A. (1974). Narcotic receptor sites in morphine dependent rats. *Nature*, 248, 61-63.
- Koelle, G.B., Koelle, E.S. & Friedenwald, J.S. (1950). The effect of inhibition of specific and non-specific cholinesterase on the motility of the isolated ileum. *J. Pharmac. exp. Ther.*, 100, 180-191.
- Korf, J., Bunney, S. & Aghajanian, G.K. (1974). Noradrenergic neurons: morphine inhibition of spontaneous activity. *Europ. J. Pharmac.*, 25, 165-169.
- Kosterlitz, H.W. & Hughes, J. (1975). Some thoughts on the significance of enkephalin, the endogenous ligand. *Life Sci.*, 17, 91-96.

- Kosterlitz, H.W., Leslie, F.M. & Waterfield, A. (1975). Rates of onset and offset of action of narcotic analgesics in isolated preparations. *Europ. J. Pharmac.*, 32, 10-16.
- Kosterlitz, H.W., Lord, J.A.H. & Watt, A.J. (1972). Morphine receptor in the myenteric plexus of the guinea-pig ileum. In Agonist and Antagonist Actions of Narcotic Analgesic Drugs. eds. Kosterlitz, H.W., Collier, H.O.J. & Villareal, J.E. pp. 45-61. Baltimore: University Park.
- Kosterlitz, H.W. & Lydon, R.J. (1971). Impulse transmission in the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum. *Br. J. Pharmac.*, 43, 74-85.
- Kosterlitz, H.W., Waterfield, A.A. & Berthoud, V. (1974). Assessment of the agonist and antagonist properties of narcotic analgesic drugs by their actions on the morphine receptor in the guinea pig ileum. *Adv. Psychopharmac.*, 8, 319-334.
- Kosterlitz, H.W. & Waterfield, A.A. (1975a). In vitro models in the study of structure-activity relationships of narcotic analgesics. *A. Rev. Pharmac.*, 15, 29-47.
- Kosterlitz, H.W. & Waterfield, A. (1975b). An analysis of the phenomenon of acute tolerance to morphine in the guinea-pig isolated ileum. *Br. J. Pharmac.*, 53, 131-138.
- Kosterlitz, H.W. & Watt, A.J. (1968). Kinetic parameters of narcotic agonist and antagonists, with particular reference to N-allylnoroxymorphine (naloxone). *Br. J. Pharmac. Chemother.*, 3, 266-276.
- Kuhar, M.J., Pert, C.B. & Snyder, S. (1973). Regional distribution of opiate receptor binding in monkey and human brain. *Nature*, 245, 447-450.
- Lal, H. & Numan, R. (1976). Blockade of morphine-withdrawal body shakes by haloperidol. *Life Sci.*, 18, 163-168.
- Lamotte, C., Pert, C.B. & Snyder, S. (1976). Opiate receptor binding in primate spinal cord: distribution and changes after dorsal root section. *Brain Res.*, 112, 407-412.
- Larsson, L.I., Fahrenkrug, J., Schaffalitzky de Muckadell, O., Sundler, F., Hakansson, R. & Rehfeld, J.F. (1976). Localization of vasoactive intestinal (VIP) polypeptide to central and peripheral neurons. *Proc. natl. Acad. Sci.*, 73, 3197-3200.
- Lasagna, L., DeKornfeld, T.J. & Pearson, J.W. (1964). The analgesic efficacy and respiratory effects in man of a benzormorphan "narcotic antagonist". *J. Pharmac. exp. Ther.*, 144, 12-16.

- Lewis, M.J., Costa, J.L., Jacobowitz, D.M. & Margules, D.L. (1976). Tolerance, physical dependence and opioid seeking behavior: dependence on diencephalic norepinephrine. *Brain Res.*, 107, 156-165.
- Light, A.B. (1931). Physiologic aspects of opium addiction. *J. Amer. Med. Assoc.*, 96, 823-825.
- Linseman, M.A. (1978). Hippocampal unit responses to low doses of morphine and naloxone in drug-naive and morphine-dependent rats. *Neurosci., Abstr.*, 4, 134.
- Loh, H.H., Cho, T.M., Wu, Y.C. & Way, E.L. (1974). Stereospecific binding of narcotics to brain cerebroside. *Life Sci.*, 14, 2231-2245.
- Lord, J.A.H., Waterfield, A.A., Hughes, J. & Kosterlitz, H.W., (1977). Endogenous opioid peptides; multiple agonists and receptors. *Nature*, 267, 495-499.
- MacDonald, R.L. & Nelson, P.G. (1978). Specific-opiate-induced depression of transmitter release from dorsal root ganglion cells in culture. *Science*, 199, 1449-1451.
- Martin, W.R. (1971). Drug dependence. In Drill's Pharmacology in Medicine. ed. DiPalma, J.R. pp. 362-378. McGraw-Hill.
- Martin, W.R., Eades, C.G., Thompson, J.A., Huppler, R.E. & Gilbert, E. (1976). The effects of morphine- and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dog. *J. Pharmac. exp. Ther.*, 197, 517-532.
- Martin, W.R., Fraser, H.F., Gorodetzky, C.W. & Rosenberg, D.E. (1965). Studies of the dependence-producing potential of the narcotic antagonist 2-cyclopropylmethyl-2'-hydroxy-5,9-dimethyl-6,7-benzomorphan (cyclazocine, WIN-20,740, ARC II-C-3). *J. Pharmac. exp. Ther.*, 150, 426-436.
- Mason, T.H. & Hamby, W.B. (1948). Relief of morphine addiction by prefrontal lobotomy. *J. Amer. Med. Assn.*, 136, 1039-1040.
- McClung, R. & Dafny, N. (1978). Single unit recording from the parafascicular nucleus of the thalamus in naive and morphine dependent rats. *Fedn. Proc.*, 37, 827.
- McKnight, A.T., Sosa, R.P., Hughes, J. & Kosterlitz, H.W. (1978). Biosynthesis and release of enkephalins. In Characteristics and Functions of Opioids. eds. van Ree, J.M. and Terenius, L. pp. 259-270. Amsterdam: Elsevier.
- Michne, W.F., Pierson, A.K. & Albertson, N.F. (1974). Ketocyclazocine: A narcotic with a new activity profile. Presented to Committee on Problems of Drug Dependence, National Research Council, Mexico City.

- Millett, L.B. & Woods, L.A. (1956). The distribution and fate of morphine in the non-tolerant and tolerant monkey. *J. Pharmac. exp. Ther.*, 116, 77-83.
- Minneman, K.P. & Iverson, L.L. (1977). Morphine selectively blocks dopamine-stimulated cyclic AMP formation in the rat neostriatal slices. *Br. J. Pharmac.*, 59, 480-481P.
- Mushlin, B.E., Griffel, P. & Cochin, J. (1975). Blockade of the development of physical dependence to morphine by concurrent naloxone administration. *Fedn. Proc.*, 34, 735.
- Nathanson, J.A. (1977). Cyclic nucleotides and nervous system function. *Physiol. Rev.*, 57, 157-256.
- Nicoll, R.A., Siggins, G.R., Ling, N., Bloom, F.E. & Guillemin, R. (1977). Neuronal actions of endorphin and enkephalins among brain regions: a comparative microiontophoretic study. *Proc. natl. Acad. Sci.*, 74, 2584-2588.
- Nilsson, G., Larsson, L.I., Hakansson, R., Brodin, E., Pernow, B. & Sundler, F. (1975). Localization of substance-P-like immunoreactivity in mouse gut. *Histochem.*, 43, 97-99.
- Nishi, S. & North, R.A. (1973a). Intracellular recording from the guinea pig ileum. *J. Physiol.*, 231, 471-491.
- Nishi, S. & North, R.A. (1973b). Presynaptic action of noradrenaline in myenteric plexus. *J. Physiol.*, 231, 29-30P.
- Norberg, K.A. (1964). Adrenergic innervation of the intestinal wall studied by fluorescence microscopy. *Int. J. Neuropharmac.*, 3, 379-382.
- North, R.A. (1973). The calcium-dependent slow, after-hyperpolarization in myenteric plexus neurones with tetrodotoxin-resistant action potentials. *Br. J. Pharmac.*, 49, 709-711.
- North, R.A. (1979a). Opiates, opioid peptides and single neurones. *Life Sci.*, 24, 1527-1546.
- North, R.A. (1979b). Mechanism of action of opiates on single myenteric neurons. In *Mechanisms of Pain and Analgesic Compounds*. eds. Beers, R.F. and Bassett, E.G. pp. 373-381. New York: Raven Press.
- North, R.A., Katayama, Y. & Williams, J.T. (1979). On the mechanism and site of action of enkephalin on single myenteric neurons. *Brain Res.*, 165, 67-77.
- North, R.A. & Tonini, M. (1977). The mechanism of action of narcotic analgesics in the guinea-pig ileum. *Br. J. Pharmac.*, 61, 541-549.

- North, R.A. & Williams, J.T. (1976). Enkephalin inhibits firing of myenteric neurones. *Nature*, 264, 460-461.
- North, R.A. & Williams, J.T. (1977). Extracellular recording from the guinea-pig myenteric plexus and the action of morphine. *Europ. J. Pharmac.*, 45, 23-33.
- North, R.A. & Vitek, L.V. (1980). A study of the role of cyclic AMP in the depression by opiates and opioid peptides of excitatory junction potentials in the mouse vas deferens. *Br. J. Pharmac.*, (submitted).
- North, R.A. & Zieglgänsberger, W. (1978). Opiate withdrawal signs in single myenteric neurones. *Brain Res.*, 144, 208-211.
- Ohkawa, H. & Prosser, C. (1972). Electrical activity in myenteric and submucous plexuses of cat intestine. *Amer. J. Physiol.*, 222, 1412-1419.
- Oliveras, J.L., Besson, J.M., Guilbaud, G. & Liebeskind, J.C. (1974). Behavioral and electrophysiological evidence of pain inhibition from midbrain stimulation in the cat. *Exp. Brain Res.*, 20, 32-44.
- Opmeer, F.A. & van Ree, J.M. (1978). Quantification of the *in vitro* induced tolerance to morphine of the isolated guinea-pig ileum. *Neuropharmac.*, 17, 887-890.
- Paton, W.D.M. (1957). The action of morphine and related substances on contraction and on acetylcholine output of coaxially stimulated guinea-pig ileum. *Br. J. Pharmac.*, 11, 119-127.
- Pert, A. (1976). Behavioral pharmacology of D-alanine<sup>2</sup>-methionine enkephalin amide and other long-acting opiate peptides. In Opiates and Endogenous Opioid Peptides. ed. Kosterlitz, H.W. pp. 87-94. Amsterdam: North Holland.
- Pert, A. (1978). Central sites involved in opiate actions. In The Bases of Addiction. ed. Fishman, J. pp. 299-332. Berlin: Dahlem Konferenzen.
- Pert, A. & Yaksh, T. (1974). Sites of morphine induced analgesia in the primate brain: relation to pain pathways. *Brain Res.*, 80, 135-140.
- Pert, C.B., Kuhar, M.J. & Snyder, S. (1975). Autoradiographic localization of the opiate receptor in rat brain. *Life. Sci.*, 16, 1849-1854.
- Pert, C.B., Pasternak, G. & Snyder, S.H. (1973). Opiate agonists and antagonists discriminated by receptor binding in brain. *Science*, 182, 1359-1361.
- Pert, C.B., Snowman, A.M. & Snyder, S.H. (1974). Localization of opiate receptor binding in synaptic membranes of rat brain. *Brain Res.*, 70, 184-188.

- Pert, C.B. & Snyder, S.H. (1973). Opiate receptor: Demonstration in nervous tissue. *Science*, 179, 1011-1014.
- Peters, R.I. & Klemm, W.R. (1977). Electrophysiological signs of differential tolerance development to morphine in selected areas of the rabbit brain. *Life Sci.*, 20, 85-94.
- Przewlocki, R., Hölt, V., Duka, T.H., Kleber, G., Gramsch, C.H., Haarman, I. & Herz, A. (1979). Long-term morphine treatment decreases endorphin levels in rat brain and pituitary. *Brain Res.*, 174, 357-361.
- Ramwell, P.W. & Shaw, J.E. (1970). Biological significance of prostaglandins. *Progr. Hormone Res.*, 26, 139-187.
- Rance, M.J. & Dickens, J.M. (1978). The influence of drug-receptor kinetics on the pharmacological and pharmacokinetic profiles of buprenorphine. In Characteristics and Function of Opioids. eds. van Ree, J. and Terenius, L. pp. 65-66. Amsterdam: Elsevier.
- Ritchie, J.M. (1975). Central nervous system stimulants. In The Pharmacological Basis of Therapeutics. eds. Goodman, L.S. and Gilman, A. pp. 367-378. New York: Macmillan.
- Ritzmann, R.F. & Tabakoff, B. (1976). Dissociation of alcohol tolerance and dependence. *Nature*, 263, 418-420.
- Robbins, S.L. (1974). Pathologic Basis of Disease. Philadelphia: Saunders.
- Ryan, J.J., Parker, C.W. & Williams, R.C. Jr. (1972).  $\gamma$ -Globulin binding of morphine in heroin addicts. *J. Lab. Clin. Med.*, 80, 155-164.
- Sanfacon, G. & Lambreque, G. (1977). Acetylcholine antirelease effect of morphine and its modification by calcium. *Psychopharmac.*, 55, 151-156.
- Sato, T., Takayanagi, I. & Takagi, K. (1973). Pharmacological properties of electrical activities obtained from neurons in Auerbach's plexus. *Jap. J. Pharmac.*, 23, 665-671.
- Sato, T., Takayanagi, I. & Takagi, K. (1974). Effects of acetylcholine releasing drugs on electrical activities obtained from Auerbach's plexus in the guinea-pig ileum. *Jap. J. Pharmac.*, 24, 447-451.
- Sato, M., Nakamura, N. & Takagi, H. (1971). Effect of morphine on bradykinin-induced unitary discharges in the spinal cord of the rabbit. *Eur. J. Pharmac.*, 16, 245-247.
- Sato, M., Zieglgänsberger, W., Fries, W. & Herz, A. (1974). Opiate agonist-antagonist interaction at cortical neurones on naive and tolerant dependent rats. *Brain Res.*, 82, 378-382.



- Satoh, M., Zieglgänsberger, W. & Herz, A. (1975). Interaction between morphine and putative excitatory neurotransmitters in cortical neurones in naive and tolerant rats. *Life Sci.*, 17, 75-80.
- Satoh, M., Zieglgänsberger, W. and Herz, A. (1976). Actions of opiates upon single unit activity in the cortex of naive and tolerant rats. *Brain Res.*, 115, 99-110.
- Sawynok, J. & Jhamandas, K.H. (1976). Inhibition of acetylcholine release from cholinergic nerves by adenosine, adenine nucleotides and morphine: antagonism by theophylline. *J. Pharmac. exp. Ther.*, 197, 379-390.
- Schaumann, W. (1957). Inhibition by morphine of the release of acetylcholine from the intestine of the guinea-pig. *Br. J. Pharmac.*, 12, 115-118.
- Schofield, G.C. (1968). Anatomy of muscular and neural tissues in the alimentary canal. In *Handbook of Physiology*, Sec. 6, vol. IV, ed. Code, C.F. pp. 1579-1628. Baltimore: Williams and Wilkins.
- Schulz, R. & Cartwright, C. (1976). Sensitization of the smooth muscle by prostaglandin  $E_1$  contributes to reversal of drug-induced inhibition of the guinea-pig ileum. *Naunyn-Schmiedeberg's Arch. Pharmac.*, 294.
- Schulz, R., Cartwright, C. & Goldstein, A. (1974). Reversibility of morphine tolerance and dependence in guinea pig brain and myenteric plexus. *Nature*, 251, 329-331.
- Schulz, R. & Goldstein, A. (1973). Morphine tolerance and supersensitivity to 5-hydroxytryptamine in the myenteric plexus of the guinea-pig. *Nature*, 244, 168-170.
- Schulz, R. & Herz, A. (1976a). Aspects of opiate dependence in the myenteric plexus of the guinea-pig. *Life Sci.*, 19, 1117-1128.
- Schulz, R. & Herz, A. (1976b). The guinea-pig ileum as an *in vitro* model to analyze dependence liability of narcotic drugs. In *Opiates and Endogenous Opiate Peptides*. ed. Kosterlitz, H.W. pp. 319-326. Amsterdam: Elsevier.
- Sharma, S.K., Klee, W.A. & Nirenberg, M. (1975). Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. *Proc. natn. Acad. Sci.*, 72, 3092-3096.
- Sharma, S.K., Klee, W.A. & Nirenberg, M. (1977). Opiate-dependent modulation of adenylate cyclase. *Proc. natn. Acad. Sci.*, 74, 3365-3369.
- Shoham, S. & Weinstock, M. (1974). The role of supersensitivity to acetylcholine in the production of tolerance to morphine in stimulated guinea-pig ileum. *Br. J. Pharmac.*, 52, 597-603.

- Snyder, S.H., Pasternak, G.W. & Pert, C.B. (1975). Opiate receptor mechanisms. In Handbook of Psychopharmacology. eds. Iverson, L.L. Iverson, S. and Snyder, S.H. pp. 329-360. New York: Plenum Press.
- Spiehl, V.R. & Paalzow, L. (1979). Naloxone antagonism of phenoxybenzamine antinociception in the mouse tail stimulation test. *Life Sci.*, 24, 2125-2132.
- Svensson, T.H., Bunney, B.S. and Aghajanian, G.K. (1975). Inhibition of both noradrenergic and serotonergic neurons in brain by the  $\alpha$ -adrenergic agonist clonidine. *Brain Res.*, 92, 291-306.
- Swain, H.H. & Seevers, M.H. (1974). Evaluation of new compounds for morphine-like physical dependence in the rhesus monkey. Presented to Committee on Problems of Drug Dependence, National Research Council, Mexico City.
- Takagi, H., Doi, T. & Akaike, A. (1976). Microinjection of morphine in the medial part of the bulbar reticular formation in rabbit and rat: inhibitory effects on lamina V cells of spinal dorsal horn and behavioral analgesia. In Opiates and Endogenous Opioid Peptides. ed, Kosterlitz, H.W. pp. 191-199. Amsterdam: Elsevier.
- Takagi, K. & Takayanagi, I. (1972). Effect of N ,2'-O-dibutyryl-3',5'-adenosine monophosphate, 3',5'-cyclic adenosine monophosphate and adenosine triphosphate on acetylcholine output from cholinergic nerves in guinea-pig ileum. *Jap. J. Pharmac.*, 22, 33-36.
- Takayanagi, I., Sato, T. & Takagi, K. (1974). Action of 5-hydroxytryptamine on electrical activity of Auerbach's plexus in the ileum of the morphine dependent guinea-pig. *Europ. J. Pharmac.*, 27, 252-254.
- Taxi, J. (1965). Contribution a l'etude des connexions des neurones moteurs du systeme nerveux autonome. *Annls. Sci. nat. (Zool.)*, 7, 413-674.
- Teitelbaum, H., Catravas, G.N. & McFarland, W.L. (1974). Reversal of morphine tolerance after medial thalamic lesions in the rat. *Science*, 185, 449-451.
- Thesleff, S. (1960). Effects of motor innervation on the chemical sensitivity of skeletal muscle. *Physiol. Rev.*, 40, 734-752.
- Urcu, G. & Liebeskind, J. (1979). Electrophysiological indices of opiate action in awake and anesthetized rats. *Brain Res.*, 161, 162-166.
- Van Inwegen, R.G., Strada, S.J. & Robison, G.A. (1975). Effects of prostaglandin and morphine on brain adenyl cyclase. *Life Sci.*, 16, 1875-1876.
- Van Nueten, J.M., Janssen, A.J. & Fontaine, J. (1976a). Unexpected reversal effects of naloxone on the guinea-pig ileum. *Life Sci.*, 18, 803-809.

- Van Nueten, J.M., Janssen, P.A. & Fontaine, J. (1976b). Naloxone reverses inhibitory effects of fatigue and of compounds not related to narcotic analgesics in the guinea pig ileum. *Arch. Int. Pharmacodyn. Ther.*, 220, 349-350.
- Wainer, B.H., Wung, W.E., Connors, M. & Rothberg, R.M. (1979). The specificity of antimorphine and antimeperidine antibodies and their reactivity with opioid peptides. *J. Pharmac. exp. Ther.*, 208, 498-506.
- Walter, R., Ritzman, R.F., Bhargava, H.N. & Flexner, L.B. (1979). Prolyl-leucyl-glycinamide, cyclo (leucylglycine) and derivatives block development of physical dependence on morphine in mice. *Proc. natl. Acad. Sci.*, 76, 518-520.
- Walter, R., Ritzman, R.F., Bhargava, H.N., Rainbow, T.C., Flexner, L.B. & Krivoy, W.A. (1978). Inhibition by Z-Pro-D-Leu of development of tolerance to and physical dependence on morphine in mice. *Proc. natl. Acad. Sci.*, 75, 4573-4576.
- Ward, A. & Takemori, A.E. (1976). Effect of 6-hydroxydopamine and 5,6-dihydroxytryptamine on the response of the coaxially stimulated guinea-pig ileum to morphine. *J. Pharmac. exp. Ther.*, 199, 124-130.
- Waterfield, A.A., Hughes, J. & Kosterlitz, H.W. (1976). Cross tolerance between morphine and methionine enkephalin. *Nature*, 260, 624-625.
- Waterfield, A., Smokcum, R.W., Hughes, J., Kosterlitz, H.W. & Henderson, G. (1977). *In vitro* pharmacology of the opioid peptides, enkephalins and endorphins. *Europ. J. Pharmac.*, 43, 107-116.
- Way, E.L., Loh, H.H. & Shen, F.H. (1969). Simultaneous quantitative assessment of morphine tolerance and physical dependence. *J. Pharmac. exp. Ther.*, 167, 1-8.
- Wei, E., Loh, H.H. & Way, E.L. (1973). Brain sites of precipitated abstinence in morphine-dependent rats. *J. Pharmac. exp. Ther.*, 185, 108-115.
- Wei, E., Sigel, S.S.R., Loh, H.H. & Way, E.L. (1975). Central sites of naloxone-precipitated shaking in the anesthetized morphine-dependent rat. *J. Pharmac. exp. Ther.*, 195, 480-487.
- Welman, W. & Schwabe, U. (1973). Effects of prostaglandin E<sub>1</sub>, E<sub>2</sub> and E<sub>3</sub> on cyclic AMP levels in brain *in vivo*. *Brain Res.*, 59, 371-378.
- Werner, V., Starke, K. & Schuman, H.J. (1972). Actions of clonidine and 2-(-2 methyl-6-cyclohexylamino)-2-oxazoline on postganglionic autonomic nerves. *Arch. Int. Pharmacodyn. Ther.*, 195, 282-290.
- Wesche, D., Höllt, V. & Herz, A. (1977). Radioimmunoassay of enkephalin. *Nauyn-Schmiedeberg's Arch. Pharmac.*, 301, 79-82.

- Wilkening, D., Mishra, R.K. & Makman, M.H. (1976). Effects of morphine on dopamine-stimulated adenylate cyclase and on cyclic GMP formation in primate brain amygdaloid nucleus. *Life Sci.*, 19, 1129-1138.
- Williams, J.T. & North, R.A. (1978a). Excitation of myenteric neurons by substance P. *Fedn. Proc.*, 37, 482.
- Williams, J.T. & North, R.A. (1978b). Inhibition of firing of myenteric neurones by somatostatin. *Brain Res.*, 155, 165-168.
- Williams, J.T. & North, R.A. (1979a). Effects of endorphins on single myenteric neurons. *Brain Res.*, 165, 57-65.
- Williams, J.T. & North, R.A. (1979b). Vasoactive intestinal polypeptide excites neurones of the myenteric plexus. *Brain Res.*, 175, 174-177.
- Williams, J.T., Katayama, Y. & North, R.A. (1979). The action of neurotensin on single myenteric neurones. *Europ. J. Pharmac.*, 59, 181-186.
- Wood, J.D. (1970). Electrical activity from single neurons in Auerbach's plexus. *Amer. J. Physiol.*, 219, 159-169.
- Wood, J.D. (1975). Neurophysiology of Auerbach's plexus and control of intestinal motility. *Physiol. Rev.*, 52, 307-324.
- Wood, J.D. & Mayer, C.J. (1978). Slow synaptic excitation mediated by serotonin in Auerbach's plexus. *Nature*, 276, 836-837.
- Woods, L.A. (1954). Distribution and fate of nalorphine in dog and rat. *J. Pharmac. exp. Ther.*, 112, 158-175.
- Yaksh, T.L. & Rudy, T.A. (1976). Analgesia mediated by a direct spinal action of narcotics. *Science*, 192, 1357-1358.
- Yano, I., Nishino, H. & Murano, T. (1979). Antagonism by naloxone of tolerance and dependence in mice given a single dose of morphine. *Jap. J. Pharmac.*, 29, 357-366.
- Yokoyama, S. (1966). Aktions-potentiale der ganglienzelle des Auerbachschen plexus in kaninchendunndarm. *Pfluegers Arch.*, 288, 95-102.
- Zieglgänsberger, W. & Bayerl, H. (1976). The mechanism of inhibition of neuronal activity by opiates in the spinal cord of cats. *Brain Res.*, 115, 111-128.
- Zieglgänsberger, W., Siggins, G., French, E. & Bloom, F. (1978). Effects of opioids on single unit activity. In Characteristics and Function of Opioids. eds. van Ree, J.M. and Terenius, L. pp. 75-81. Amsterdam: Elsevier.

# APPROVAL SHEET

The dissertation submitted by Peter J. Karras has been read and approved by the following committee:

Dr. R. Alan North, Director  
Associate Professor, Pharmacology, Loyola

Dr. A. G. Karczmar  
Chairman of Pharmacology, Loyola

Dr. H. Proudfit  
Associate Professor, Pharmacology, Univ. of Illinois

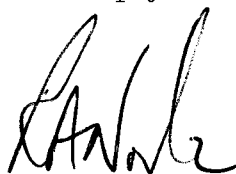
Dr. B. Wainer  
Assistant Professor, Pharmacology, Univ. of Chicago

Dr. R. D. Wurster  
Associate Professor, Physiology, Loyola

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to context and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmacology

Date June 23-80



Director's Signature